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Clinical and Experimental

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STUDIES ON COPPER METABOLISM

VIII. The Correlation Between the Serum Copper Level and Various Serum Protein Fractions

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COPPER METABOLISM

IN A previous paper in this series[1] it was noted that an increase in the plasma copper level occurs in a variety of unrelated conditions which include pregnancy, chronic infections, acute leukemia, Hodgkin's disease, iron deficiency anemia, and aplastic anemia. The significance of hypercupremia is unknown. Although copper is intimately concerned in erythropoiesis,[2, 3] there is no evidence to indicate that hypercupremia in human subjects is related in any way to alterations in erythropoiesis.[4]

It has been demonstrated recently that the copper in serum is bound to an α-globulin.[5, 6] It is also known that severe bodily injury of almost any type is associated with an increase in α-globulins, as well as with an increase in plasma fibrinogen, hexosamines, polysaccharides, and mucoproteins.[7, 8] Similar alterations in plasma proteins have been observed in pregnancy.[7, 8] Since, in general, it is under these circumstances that hypercupremia is also observed,[1, 9, 10] it was thought to be of interest to determine if there is a close correlation between hypercupremia and the above-mentioned alterations in protein metabolism. Such studies might aid in understanding the significance of hypercupremia in disease.

It is the purpose of this report to present data concerning the correlation between the plasma or serum copper level and the amounts of α-globulin and other protein fractions as measured electrophoretically in normal and diseased human subjects. Studies were also made concerning the correlation of the copper levels and the chemically determined albumin, globulin and mucoprotein fractions.

In addition to the observations on human subjects, data dealing with the correlation between plasma copper levels and the various plasma protein fractions in normal, hypercupremic and hypocupremic rats will be presented.

A preliminary report of this work has appeared.[11]

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*National Research Council Fellow in the Medical Sciences, 1949-1951.

METHODS

The protein determinations and electrophoretic analyses were performed on sera obtained from normal human subjects, patients in the last trimester of pregnancy, and patients with chronic infections and leukemia. In order to obtain human blood low in copper,[10] umbilical vein blood was used. The studies in rats were made on plasma obtained from normal adult male Sprague-Dawley rats, and from rats five days after the intramuscular injection of 0.1 ml. of turpentine (Rexall, Oil of Turpentine, U.S.P.) or of a culture of *Staphylococcus aureus* organisms. Weanling rats were made copper-deficient by feeding a milk diet low in copper and supplemented with iron.

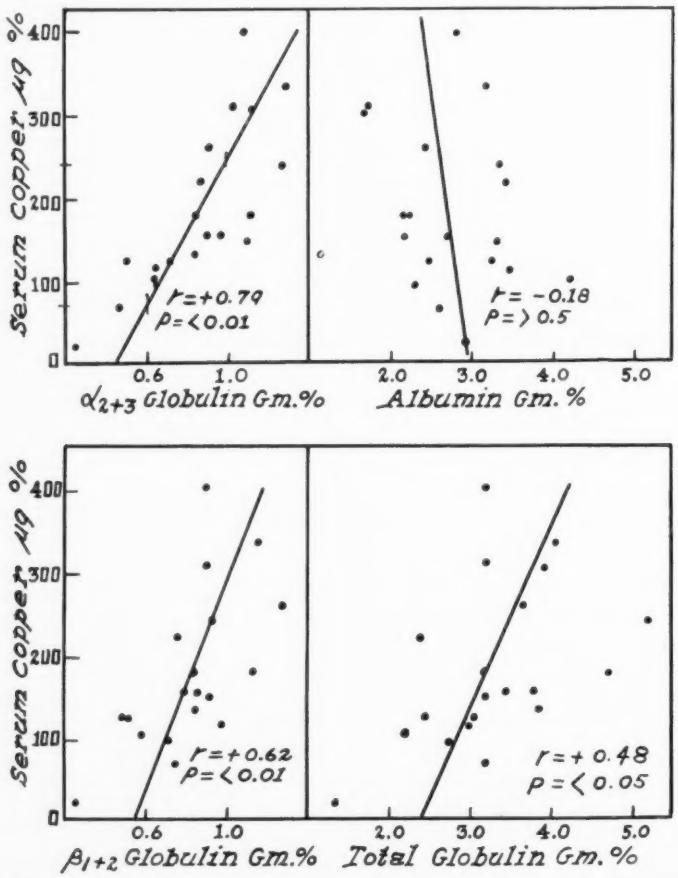

Fig. 1.—The relationship between copper concentration and electrophoretically determined protein fractions in human sera. The diagonal lines are those which best fit and were calculated by the method of least squares. *r* refers to the correlation coefficient. *P* refers to the probability with which the correlation coefficient could occur by chance.

The electrophoretic analyses were made on human serum and on rat plasma at 1°C. in a Tiselius apparatus equipped with a Longsworth schlieren scanning device. The samples were dialyzed forty-eight hours against diethylbarbiturate (veronal) buffer at pH 8.4 to 8.6 and at an ionic strength of 0.1. The protein concentration was about 1.5 per cent. Only descending patterns were used. The values in grams per cent are calculated from the percentage for each component of the total refractive increment and the total protein content of the serum, with the factor 6.7 × protein N. Total serum protein, albumin, and globulin were

determined by the biuret method[12] with the modification of Kingsley.[13] The method of Winzler and associates[14] was used for the measurement of plasma mucoproteins. The copper determinations were carried out as described in the first paper of this series.[15]

RESULTS

Human Subjects.—Electrophoretic analyses were made on 19 subjects with serum copper levels ranging from 24 to 402 μg per cent. The electrophoretic components were identified in the usual manner as albumin, α, β and γ-globulins. Since several components were present in each of the globulin fractions, these were called α_1, α_2, α_3, β_1, β_2, etc. In most instances, complete resolution of the subfractions could not be achieved, and we have considered the α_2 and α_3 together and the β_1 and β_2 as single fractions.

The results presented in Fig. 1 show that there was a high correlation between the serum copper level and the $\alpha_2 + \alpha_3$ globulin fraction (correlation coefficient $r = +0.79$) and between the serum copper and the $\beta_1 + \beta_2$ globulin fraction ($r = +0.62$). No significant degree of correlation was observed between the serum copper level and the total protein, the albumin, α_1-globulin or γ-globulin fractions.

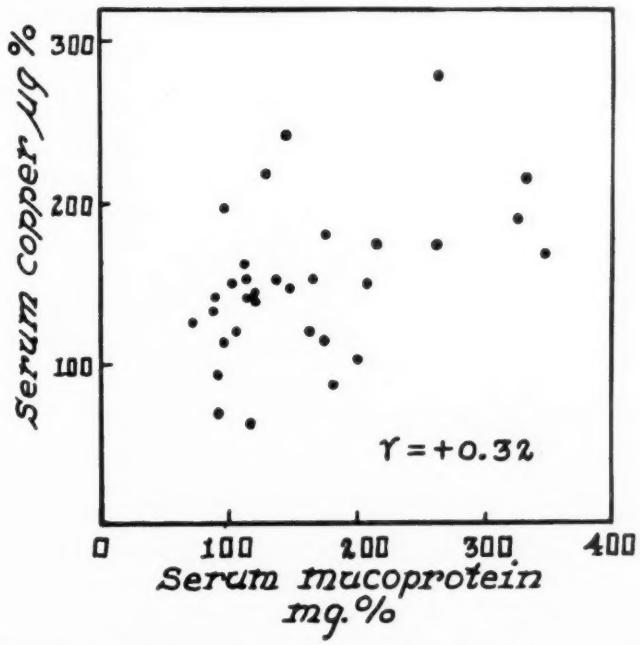

Fig. 2.—The relationship between the copper concentration and the mucoprotein concentration in human sera.

Simultaneous determinations of serum copper and total mucoproteins were made on sera obtained from 33 human subjects. The results, given in Fig. 2, reveal no significant degree of correlation. In a few patients serial determinations of copper and mucoproteins were made throughout the course of an illness. The results of such a study in a patient with active rheumatoid

arthritis treated with adrenocorticotrophic hormone (ACTH) are presented in Fig. 3. During the initial course of therapy the joint pain, tenderness, and swelling subsided markedly and fever disappeared. Concomitantly with the clinical improvement there was a decrease in the serum copper and mucoprotein levels. However, as relapses occurred with interruption of ACTH therapy there was an increase in the copper level unassociated with an increase in the mucoprotein level. During the second course of hormone therapy, clinical improvement was less striking, and there was only a slight decrease in the degree of hypercupremia. When therapy was stopped, there was again an increase in the levels of both copper and mucoproteins.

Studies were made to determine if a correlation could be observed between the serum copper level and the total protein, albumin, and globulin, as determined by a standard clinical fractionation method (21.5 per cent sodium sulfate);[10] these were carried out in 110 subjects with serum copper levels ranging from 63 to 291 μg per cent. The results, shown in Table I, fail to reveal any correlation.

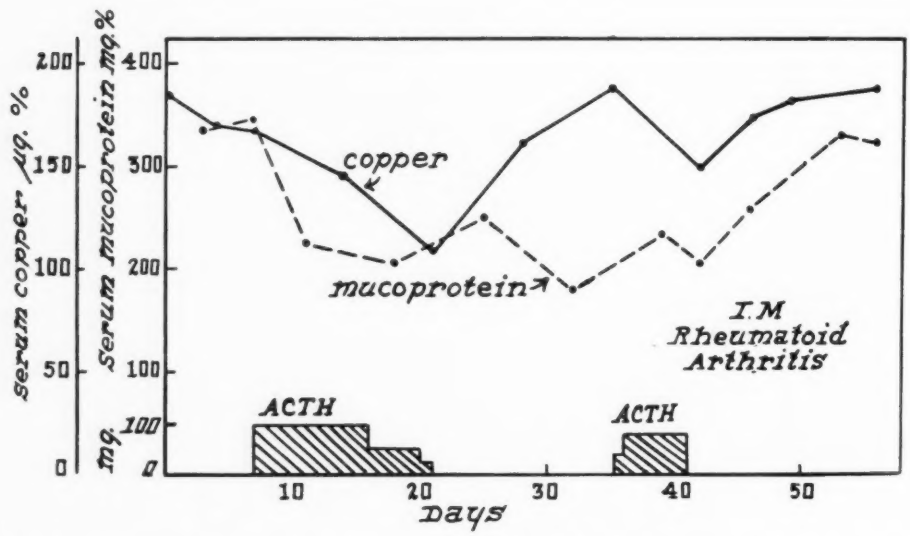

Fig. 3.—Alterations in the copper and mucoprotein concentrations in the serum of a patient (I.M.) with active rheumatoid arthritis treated with ACTH.

Albino Rats.—The correlation between copper and protein fractions determined electrophoretically was investigated in 170 rats with plasma copper values ranging from 26 to 234 μg per cent. The analyses were performed on plasma pooled from ten rats. The results are presented in Fig. 4. In contrast to the human data, the components having the same mobility as the human $\alpha_2 + \alpha_3$ globulin showed no significant correlation ($r = +0.21$) with the plasma copper. The fraction corresponding to α_1-globulin showed a high correlation ($r = +0.76$) with the plasma copper, although the greatest correlation ($r = +0.80$) was observed with the fraction corresponding to the human $\beta_1 + \beta_2$

globulin fraction. As in the study in man, there was a negative correlation with the albumin fraction and no significant correlation with the γ-globulins.

DISCUSSION

The data presented indicate that there is a high degree of correlation between alterations in the quantity of the $\alpha_2 + \alpha_3$-globulin fraction and the level of copper in the serum of human subjects. The samples studied included some low in copper (umbilical vein serum), normal serum, serum from pregnant

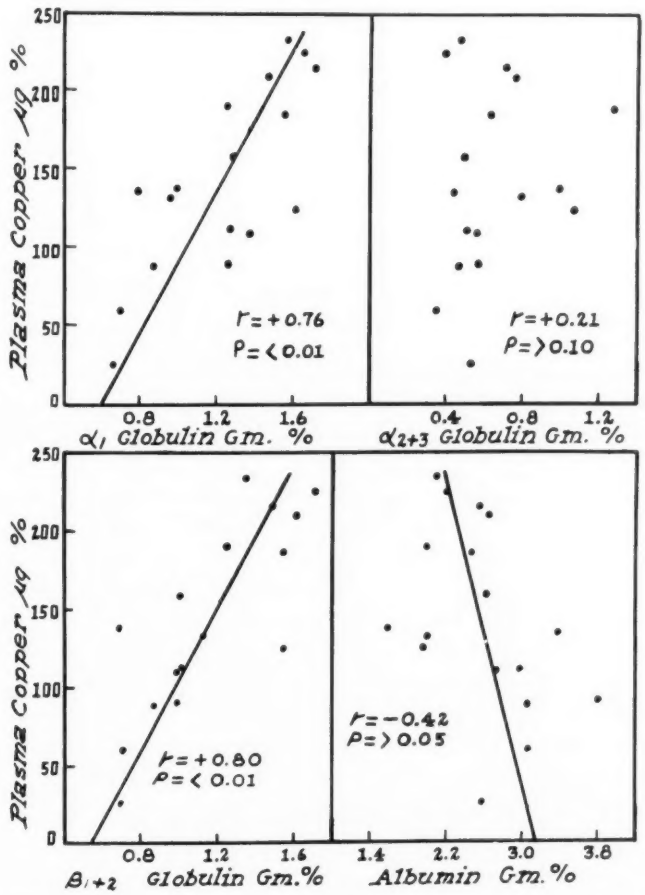

Fig. 4.—The relationship between copper concentration and electrophoretically determined protein fractions in rat plasma. Each dot represents the mean value for ten animals. The lines and symbols have the same significance as in Fig. 1.

women (high in copper), and serum from patients with infections and leukemia (high in copper). There also is good correlation between the copper level and the $\beta_1 + \beta_2$-globulin fraction. There was no significant correlation between the copper level and the amount of mucoprotein.

The studies in rats, in contrast to the data obtained in human subjects, showed good correlation between the α_1-globulin fraction and the plasma cop-

per and little or no correlation between the $\alpha_2 + \alpha_3$-globulin and copper. In the rat, as in man, a high degree of correlation was observed between the $\beta_1 + \beta_2$-globulin fraction and copper. The explanation for these species differences is not obvious. However, it must be noted that the nomenclature of Tiselius for the well defined fractions of human sera becomes very artificial when applied to animal sera.[7] Moore[16] has made a comparative study of the electrophoretic patterns of the sera of a large variety of animal species, including man. He noted considerable differences in the relative positions of the components in human sera as compared with those of rat sera.

TABLE I. THE LACK OF RELATIONSHIP BETWEEN SERUM COPPER AND TOTAL PROTEIN, ALBUMIN AND GLOBULIN FRACTIONS IN HUMAN SERA AS DETERMINED BY SALT FRACTIONATION

SERUM COPPER		NO. OF SUBJECTS	TOTAL PROTEIN* (GM. %)	ALBUMIN* (GM. %)	GLOBULIN* (GM. %)
RANGE (μG %)	MEAN (μG %)				
50-100	85	16	6.85	4.43	2.42
100-150	122	52	7.04	4.65	2.39
150-200	171	26	6.80	4.28	2.52
200-250	220	10	7.07	4.38	2.69
250-300	280	6	7.04	4.38	2.66

*Figures refer to mean value.

From these studies it would seem that the level of copper in the serum is closely associated with alterations in the serum proteins, particularly the α and β globulin fractions. Since hypercupremia occurs in human subjects under conditions associated with considerable tissue reaction, irrespective of cause, this phenomenon appears to take its place along with a number of other chemical alterations in the blood which have been referred to as "acute phase reactants."[17] Its significance is obscure. Whether the hypercupremia is the consequence of the breakdown of tissue proteins or whether it represents copper which is being mobilized to be used for a specific purpose, it is not possible to state. In the latter connection it is noteworthy, however, that Holmberg and Laurell[18, 19] have presented evidence that the serum copper protein, ceruloplasmin, functions as an oxidase (laccase) and that there is excellent correlation between the oxidase activity of serum and the copper level in umbilical vein blood, in pregnancy serum and in serum from patients with infections and other diseases associated with hypercupremia.

SUMMARY

1. Electrophoretic analyes were made on sera from 19 human subjects with serum copper levels ranging from 24 to 402 μg per cent. A high degree of correlation was observed between the copper level and the $\alpha_2 + \alpha_3$ globulin fraction (correlation coefficient, $r = +0.79$) and between the serum copper and the $\beta_1 + \beta_2$ globulin fraction ($r = +0.62$). No significant correlation was observed between the serum copper level and other protein fractions determined electrophoretically.

No significant degree of correlation was observed between the serum mucoprotein and copper levels in 33 human subjects, or between the serum

copper level and albumin, globulin, and total serum proteins determined by the usual salt fractionation method.

2. Electrophoretic analyses were made on plasma from 17 rats with plasma copper values ranging from 26 to 234 μg per cent. A high degree of correlation was observed between the plasma copper level and the α_1-globulin fraction ($r = +0.76$). The highest degree of correlation ($r = +0.80$) was found with the fraction corresponding to the $\beta_1 + \beta_2$ globulin fraction.

Acknowledgment.—We are indebted to Miss Helen Ashenbrucker for technical assistance.

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THE ABSORPTION OF IRON. RADIOIRON STUDIES IN IDIOPATHIC HEMOCHROMATOSIS, MALNUTRITIONAL CYTOSIDEROSIS, AND TRANSFUSIONAL HEMOSIDEROSIS

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INTRODUCTION

EXCESSIVE deposition of iron in the body has been found in idiopathic hemochromatosis, in malnourished South African Negro pellagrins, and in refractory anemias treated with multiple blood transfusions.

Idiopathic hemochromatosis was first described in 1889, and 436 cases of the disease had been reported by 1941.[1] It has been concluded that it is due to an inborn error in metabolism associated with the slow accumulation of iron in the body over many years.[26] More recently the Gillmans[14, 15] have shown heavy accumulations of iron in the livers of a large percentage of South African Negro pellagrins. The histologic features in some of the livers of these cases of so-called malnutritional cytosiderosis were indistinguishable from those seen in idiopathic hemochromatosis, and these authors concluded that chronic malnutrition leads to a derangement in intracellular metabolism which interferes with the normal "mucosal block,"[18] and so allows for the absorption of abnormally large quantities of iron from the gut. A third source of excessive iron deposition has been introduced with the widespread use of multiple blood transfusions in refractory anemias.[6, 7, 24, 25, 33, 34] In some of these cases the heavy hepatocellular deposits of iron have been associated with portal fibrosis, and the relation of this so-called "exogenous hemochromatosis"[25] to the idiopathic disease has been stressed by some authors.[25, 34] A feature of several of these cases has been the finding of more iron in the liver alone at autopsy than could have been present in all the blood transfusions administered.[7, 24, 33, 34] It has therefore been suggested that absorption of iron may continue to occur from the gut in spite of excessive deposits throughout the body.[33]

This investigation was undertaken to compare the pattern of iron absorption and utilization in these three types of iron excess with a group of control subjects. For this purpose 5 cases of idiopathic hemochromatosis, 5 cases of malnutritional cytosiderosis, 3 cases of transfusional hemosiderosis, and 8 control subjects were investigated under standard conditions after each had been given an equal dose of radioiron. The results obtained form the basis of this paper.

This work was assisted by a grant from the Council for Scientific and Industrial Research, South Africa.
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*Department of Medicine, University of the Witwatersrand.
**Council for Scientific and Industrial Research.

MATERIAL AND METHODS

Clinical Material.—(See Table I). The 21 cases investigated are divided into 4 groups.

Group I. Idiopathic Hemochromatosis, 5 Cases.—Diagnosis was confirmed in all cases by liver biopsy. The patients ranged in age from 24 to 60 years. There were 4 women and 1 man. Of the women, Cases 3, 4, and 5 were postmenopausal, while Case 1, who was 24 years of age, had amenorrhea of several years' duration. All showed skin pigmentation and cirrhosis of the liver, but diabetes was present in only 3. None had any significant anemia. Case 5, who was diabetic, showed typical features of hemochromatosis on liver biopsy, but deposits of iron elsewhere in the body were absent at subsequent autopsy except in the pancreas which showed fibrotic changes and minimal iron pigment. (Similar cases have been described by Herbut and Tamaki[20] and seem to represent a variant of the classical pathologic picture of idiopathic hemochromatosis.)

Group II. Malnutritional Cytosiderosis, 5 Cases.—These South African Negro subjects, ranging in age from 47 to 66 years, all had the diagnosis confirmed on liver biopsy. There were 3 women and 2 men. Each showed heavy hepatocellular and portal iron deposits together with portal fibrosis and bile duct proliferation. Three had diabetes, an uncommon feature in this condition, and two had esophageal varices. The hemoglobin values varied from 10.5 to 16.9 Gm. per cent (average 14.3 Gm. per cent).

Group III. Transfusional Hemosiderosis, 3 Cases.—Each of these cases had received more than 100 transfusions of blood and was still receiving fortnightly transfusions at the time of the study. Two showed aplasia of the bone marrow on repeated examination of sections of bone marrow aspirations taken from various sites at different times prior to this study. In Case 12 the aplasia involved all the blood elements, while in Case 13 it affected only the red cell series. In Case 12 a recent sternal puncture, taken several months after the radioiron investigation, showed a hypercellular marrow with a normoblastic reaction. The possible significance of this finding is discussed later. In the third case (No. 11), the anemia had occurred secondary to radiation therapy for chronic lymphatic leukemia, while in the other two no obvious cause for its presence could be found. In Case 11, liver biopsy showed heavy iron deposits in liver cells and in portal tracts, but thtre was no evidence of excessive fibrosis. In the other two a mild cirrhotic reaction and some bile duct hyperplasia were present, and the findings were histologically indistinguishable from those found in idiopathic hemochromatosis. All these cases showed skin pigmentation, but sugar tolerance was normal.

Group IV. Control Subjects, 8 Cases.—These cases were selected from patients undergoing medical treatment in the hospital. None suffered from anemia. They ranged from 22 to 78 years of age, and there were an equal number of men and women. Two of the women were postmenopausal. All except Cases 14 and 21 were ambulant at the time of the study. The diagnosis in each subject can be seen in Table I.

Preparation of Radioiron.—The methods on the whole were similar to those previously used by us.[1] Radioactive iron was supplied by the A.E.R.E. (Harwell, England) as Fe^{59+55} derived from the Szilard-Chalmers reaction. The activities of Fe^{59} and Fe^{55} were stated to be approximately equal in all batches obtained. Measurements in this laboratory were made only of the gamma activity of Fe^{59}, by means of a high-pressure ionization chamber, and agreed well with those made at Harwell.

Stock solutions of radioiron (ferric chloride) were prepared from these solutions. Tracer doses were then made up with activities varying from about 7 to 20 microcuries on the day of administration. Carrier iron was added to give a total quantity of 20 mg. of iron per dose. Ascorbic acid (40 mg.) was added to each dose to reduce the ferric to ferrous iron.

Administration of Radioiron.—All cases, except Case 9 in Group II, were on routine hospital diets when the studies were done. The dose of 20 mg. of iron labelled with radioiron was given orally four hours after breakfast and was diluted with water, 1 oz. of preserved fruit juice and 60 minims of dilute hydrochloric acid (B.P.). Measurements were then made to determine, when possible, the amount of radioiron not absorbed but excreted in the feces,

TABLE I

GROUP	CASE	CLINICAL FEATURES	SEX	AGE	HEMOGLOBIN IN GRAMS %	TOTAL BLOOD VOLUME IN ML.	MAXIMUM % OF ADMINISTERED DOSE IN TOTAL BLOOD	MAXIMUM IN VIVO LIVER COUNTS*	% ADMINISTERED DOSE RECOVERED FROM FECES
I Idiopathic hemochromatosis	1	Skin pigmentation, cardiac failure and amenorrhea	F	24	15.7	3,813	3.48	525.0	28.0
	2	Skin pigmentation, diabetes and testicular atrophy	M	37					
	1st dose				14.5	4,178	2.86	343.0	76.6
	2nd dose				14.8		3.50	160.0	79.9
	3	Skin pigmentation, diabetes and hepatomegaly	F	53					
	1st dose				12.6	4,112	1.84	170.0	76.0
	2nd dose				14.3		6.80	670.0	23.0
	4	Skin pigmentation, hepatomegaly and epistaxis	F	54					
	1st dose				14.4	3,135	1.79	75.6	92.0
	2nd dose				15.3		2.70	242.0	65.0
	5	Diabetes and hepatomegaly	F	66	13.0	4,500†	1.20	41.8	–
II Malnutritional cytosiderosis	6	Skin pigmentation, diabetes and hepatomegaly	M	47	12.7	6,390	1.20	0	–
	7	Diabetes and malnutrition	F	±55	15.4	4,071	0.20	0	–
	8	Hematemesis and hepatomegaly	F	63	10.5	4,500†	0.47	0	–
	9	Diabetes and hepatomegaly	M	64	15.8	4,137	2.80	91.5	–
	10	Gross malnutrition and hepatomegaly	F	66	16.9	3,493	1.20	0	–
III Transfusional hemosiderosis	11	Lymphatic leukemia with aplastic anemia secondary to x-rays. Skin pigmentation and hepatomegaly	M	44	10.1	4,500†	0.06	0	–
	12	Idiopathic refractory anemia, skin pigmentation and hepatomegaly	M	71	14.8	4,340	3.50	0	93.0
	13	Idiopathic aplastic anemia, skin pigmentation and hepatomegaly	F	49					
	1st dose				14.1	4,576	0.35	15.4	91.6
	2nd dose				9.5		0.35	24.4	91.2
IV Control subjects	14	Chronic rheumatic carditis	F	22	14.5	4,029	8.28	17.9	–
	15	Progressive bullous emphysema	M	29	15.4	5,572	4.20	0	–
	16	Diabetes	M	31	16.0	5,550	1.10	0	80.8
	17	Diabetes	F	40	16.1	5,484	7.25	0	91.6
	18	Peripheral neuritis (? infective)	M	51	15.9	4,500†	0.66	0	–
	19	Duodenal ulcer	M	56	18.8	4,013	2.99	0	79.1
	20	Diabetes	F	60	16.3	3,679	1.90	0	75.0
	21	Cirrhosis of liver and ascites	F	78	15.6	3,846	1.23	0	–

*Corrected to a standard activity of 20 microcuries.
†Assumed blood volume.

the amount taken up by various organs, but especially by the liver, and finally the percentage of the given dose present in the blood.

In three subjects in Group I a second dose of radioiron was given several months after the first one. Preliminary premedication with phosphates and fat-soluble vitamins was carried out prior to these second studies. Case 2 was given 120 grains acid sodium phosphate and 60 minims Haliver Oil, three times daily in the week preceding the administration of the radioiron and a further dose was actually given with the radioiron. Case 4 was put on a similar regime except for the use of calcium phosphate instead of acid sodium phosphate while Case 3 was given only acid sodium phosphate.

Measurements on Feces.—Where it was possible all feces passed during the first week after radioiron administration were collected and after digestion of the organic material, their activity was measured by means of a liquid sample counter of the Veall type.[29] A standard solution was made up from an aliquot of the stock solution and counts made whenever a feces sample was assayed. Direct comparison gave the activity of the feces sample in terms of the administered dose and rendered unnecessary any correction for decay or for the changing relative activities of Fe^{55} and Fe^{59}. Nursing difficulties did not allow all feces to be collected in some cases. Only the results of those cases in which the patients were cooperative enough to collect their own feces are included.

In Vivo Measurements.—These were performed over several body sites with a G.E.C. end window counter (Type E.H.M.2-Mica Window) in a lead housing. Major interest centered on the liver uptake or radioiron. In vivo measurements were started only seven days after the iron administration so as to eliminate errors introduced by the effects of unabsorbed radioiron in the intestine on the counts over other organs. Measurements were repeated about twice weekly thereafter up to periods of as long as ninety days. It was assumed that only the Fe^{59} gamma rays were being recorded as the sources being counted (e.g., liver) were at least ¾ to 1 inch from the counting window, this thickness of tissue being sufficient to absorb all beta rays from Fe^{59} and x-rays from Fe^{55}. No significant counts from skin or muscles could be found anywhere else on the body surface. Corrections for decay were therefore based on the half-life of Fe^{59}, i.e., forty-seven days.

Measurements on the Blood.—Assays were done on the blood samples which were taken several times daily for the first few days and then at longer intervals up to periods as long as four months. The activities in the blood were determined by electroplating the samples according to a slightly modified technique of Vosburgh, Flexner, and Cowie.[30] About 3 ml. of blood were taken, weighed, dry ashed, chemically processed, and plated on small copper discs in electroplating cells. The discs were then counted with a G.E.C. Counter (Type E.H.M.2-Mica window with a window thickness of 1.9 mg. per square centimeter) and the counts were compared with those due to plated standards made up by the same technique from aliquots of the stock solution from which the patient's dose was derived. As with liquid sample measurements, activities of plated samples could thus be given directly in terms of the administered dose, and the percentage of the dose appearing in the whole blood could then be calculated. For this reason blood volumes were measured in most cases using a modified Evans Blue (T-1824) technique.[17] When the measurement was not made an arbitrary volume of 4,500 ml. was assumed.

Accuracy of Measurements.—

(a) *Feces Measurements.*—Up to 10 per cent of the feces may have been lost in collection, although it is probable that the percentage was much lower than this in most cases. The error in the chemical processing and counting procedures was found to be not more than ±5 per cent.

(b) *Blood.*—Counting rates were frequently just above background, and so it was necessary to count over long periods. In each determination sufficient counts were taken to reduce the error from this source to ±5 per cent. The error introduced by the chemical processing and electroplating procedure was estimated to be ±4 per cent from the spread in activities of the 6 standard plates which were used for comparison. The fluctuations in radioiron concentration in the blood were therefore determined with a probable error of ±7 per cent.

In the subjects who received second doses of radioiron it was not found possible to correct accurately for Fe^{55} from the first dose which may have contributed toward the counts in the second series of plated samples. The results are therefore only rough approximations.

(c) *In Vivo Measurements.*—The counting error varied with the counting rate and with the length of the counting periods. It ranged from 10 to 26 per cent of the counts obtained.

RESULTS

Feces Measurements.—(See Table I.) There was a very wide individual variation in the amount of radioiron eliminated in the feces by cases in Group I (idiopathic hemochromatosis), the results ranging from 23 to 92 per cent. Unfortunately no cases in Group II (malnutritional cytosiderosis) could be included because of difficulties in the collection of their feces. It is, however, probable that the percentage eliminated was high in at least four of these cases as they showed very low blood levels of radioiron, and in vivo counts gave no evidence of organ deposition. A high percentage of radioiron was recovered from the feces of the cases of transfusional hemosiderosis (Group III), where figures ranging from 91 to 93 per cent were obtained. In the four control subjects in whom feces measurements were done figures ranging from 79 to 92 per cent were recorded.

The administration of phosphates and fat-soluble vitamins had no apparent effect in lowering intestinal absorption in the three cases of idiopathic hemochromatosis to whom they were given. In addition the presence of anemia did not seem to increase iron absorption in Case 13 (transfusional hemosiderosis) who was given a second dose of radioiron after the hemoglobin had been allowed to fall to 9.5 Gm. per cent.

It has been assumed in all cases that the iron not excreted was absorbed into the body and not retained in the gut wall, as no significant counts could be shown over the gut a week after the administration of the radioiron in Groups II, III, and IV. In Group I the low counts which were recorded could be accounted for by radioiron in the liver. (The level of counts to be expected elsewhere on the abdominal surface due to a radioiron source in the liver has been tested previously by us in cadaver experiments.[1, 5])

In Vivo Measurements.—(See Fig. 1.) In order to be able to compare the liver counts the results were corrected to what they would have been had each subject received exactly 20 microcuries. However differences in body build and in liver size and shape tend to make any comparison between cases a very rough one.

The significance of counts over organs such as the liver is also open to question. It can be argued that if as much iron is leaving the liver as is entering it then radioiron counts may still be high when the initial pool of iron in the organ is a large one of, e.g., idiopathic hemochromatosis, for if the radioactive and nonradioactive fractions become mixed until a state of equilibrium is reached then the iron leaving the liver should be made up of a very small radioactive fraction and a large inert one. If, on the other hand, the same amount of iron enters and leaves a liver with a low iron store, e.g., control

subject, then the fraction of radioactive iron going out of the liver should be a much greater one. In the studies reported here, however, the ratio of radioiron entering the liver to the quantity of inert iron already there was low (about 1 to 500) even in the control group where the liver iron is normally 400 to 500 mg.[26] If, therefore, any radioiron deposition occurred in the liver of these subjects it should have also given rise to significant counts. In actual fact, it is doubtful whether newly deposited iron is in equilibrium with the stores already

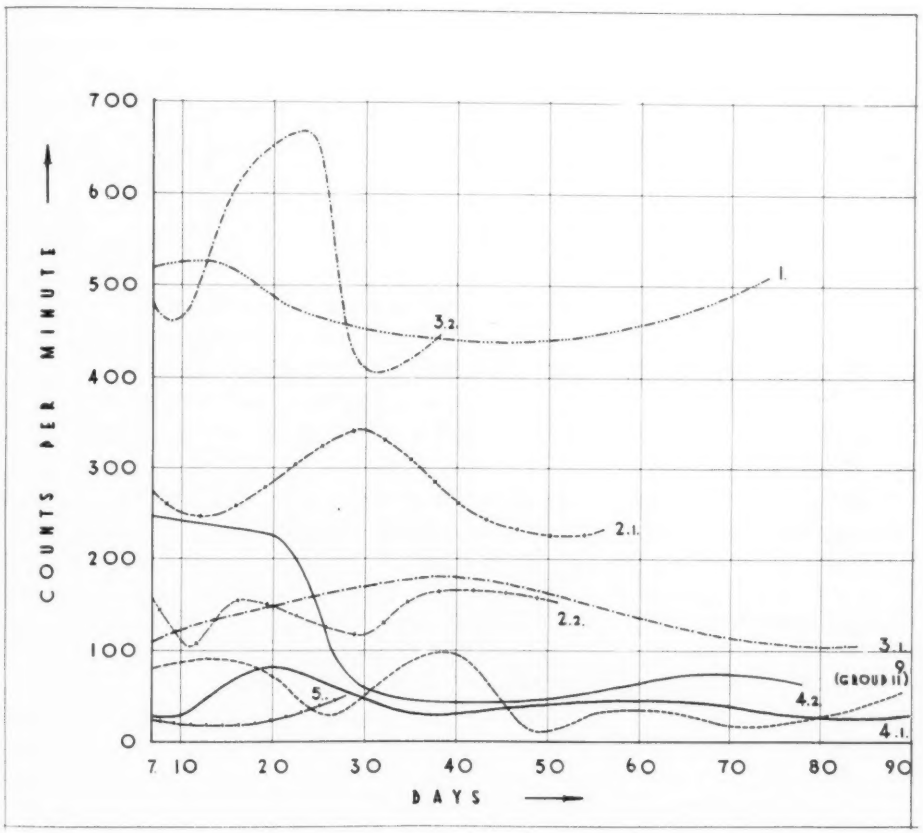

Fig. 1.—Graphs showing the in vivo counts over the livers of subjects in Group I and of Case 9 in Group II.

present in the liver, as there is suggestive evidence that the most recently absorbed iron is more readily available for utilization than older stores.[10] On this basis all in vivo counts obtained over the liver a week after the administration of radioiron have been interpreted as evidence of real deposition, while the absence of significant in vivo counts has been felt to represent absence of iron deposition, although it is also theoretically compatible with a very rapid turnover in the organ.

The only striking results were those found in Group I (idiopathic hemochromatosis). Every member of this group showed evidence of some organ

deposition of radioiron (see Fig. 1). By far the highest counts were found over the liver, although significant deposits seemed to be present in other organs such as the heart. High in vivo counts were associated with high intestinal uptakes of radioiron. In four of the five subjects with malnutritional cytosiderosis (Group II) no evidence of organ deposition of radioiron was found. In the remaining case (No. 9) significant, though not very high, counts were obtained over the liver. The results have been graphed in Fig. 1. There was no evidence of any uptake of radioiron in two of the three patients in Group III and in seven of the eight subjects in Group IV. In one of the cases with transfusional hemosiderosis (Case 13) and in one of the control group (Case 14) very low liver counts were recorded on several occasions. Case 14 had hepatomegaly secondary to previous bouts of congestive cardiac failure. The importance of her low, but probably significant, deposition of radioiron in the liver is not apparent but may have been related to hepatic congestion. It was accompanied by a very high blood uptake (8.28 per cent), and requires further study.

Blood Uptake.—The initial uptake within the first few hours was found to be in the plasma. Thereafter a slow build-up in the hemoglobin occurred over several days, and this level was then roughly maintained for periods up to 120 days. The results obtained have been graphically represented in Fig. 2 as the percentage of the administered dose in whole blood.

There was a wide variation in the maximum blood uptake in Group I (range 1.84 to 6.8 per cent). In Group II the maximum blood uptake was low, ranging from 0.2 to 2.8 per cent of the administered dose. In two cases of transfusional hemosiderosis (No. 11 and No. 13) the blood uptake was very low (0.06 and to 0.35 per cent). In one of these (No. 13) the study was repeated when the patient was anemic (hemoglobin 9.5 Gm. per cent), but similar results were obtained. Case 12 is of special interest, as the patient showed a significant level of 3.5 per cent of the administered dose in the blood in spite of the fact that sections of repeated bone marrow punctures had shown an aplasia of the marrow. Another feature was the unusually short period that the radioiron was maintained in the blood at its maximum level, falling from 3.5 to 0.7 per cent of the administered dose between the fifteenth and thirtieth days (see Fig. 2). In view of these results a further bone marrow aspiration was performed subsequent to the radioiron investigation. On this occasion the marrow was hypercellular and showed a normoblastic reaction. It therefore seems probable that Case 12 was forming blood in a normal way at the time of the radioiron investigation in spite of the previous evidence of marrow aplasia. In addition the rapid fall in the blood level of radioiron between the fifteenth and thirtieth days suggests that blood destruction was occurring at an increased rate. Evidence of hemolysis, as judged by a raised serum bilirubin, increased urinary urobilin, and reticulocytosis was, however, lacking. The appearance of radioiron in the blood of Case 12 also seemed to show that iron absorption may continue to occur in spite of heavy body stores of iron and in spite of an almost saturated serum iron level (serum iron 211 micrograms per cent; total iron binding

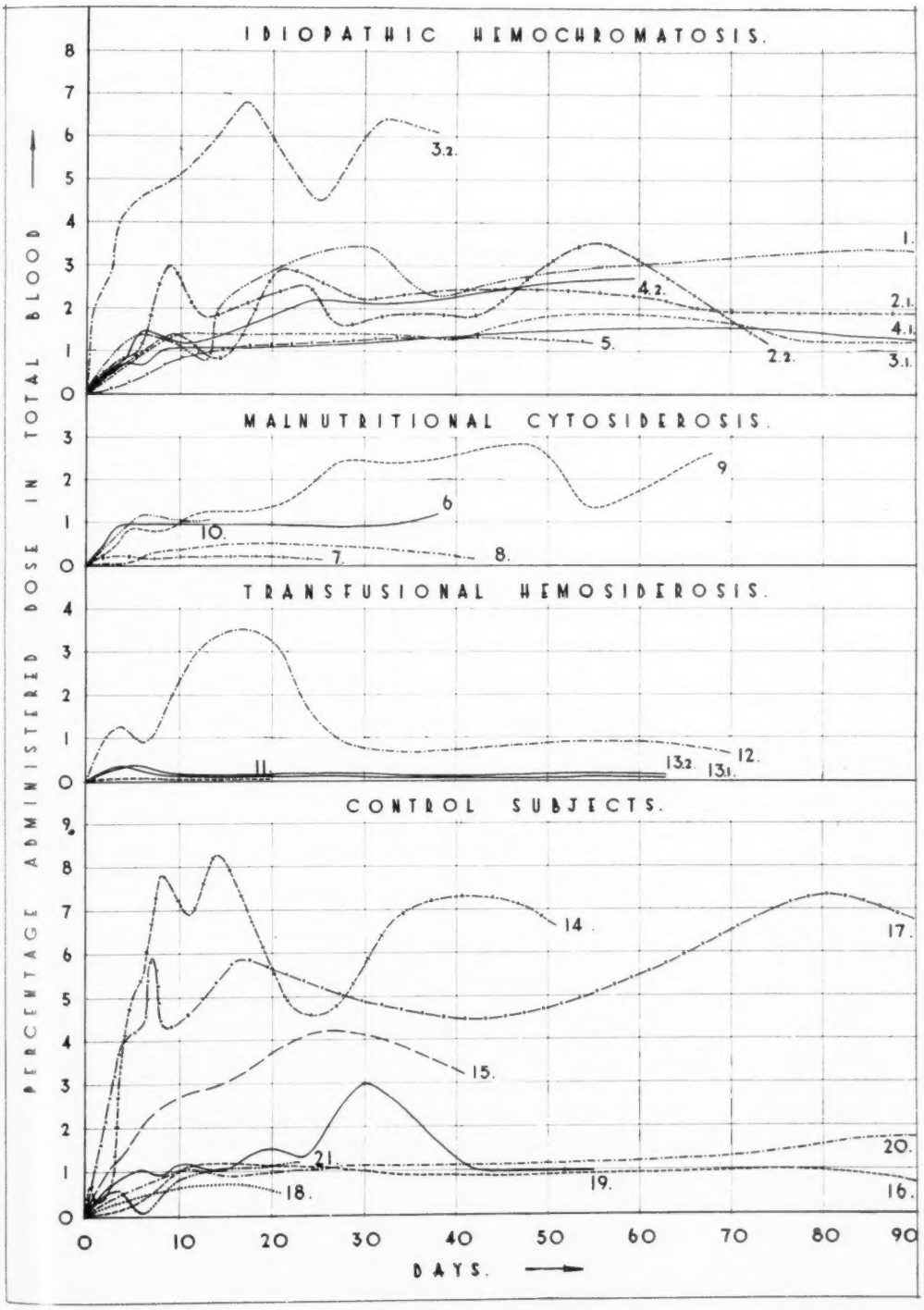

Fig. 2.—Graphs showing the percentage of the administered dose circulating in the blood in the various groups. The fluctuations in the plasma in the first few hours are not shown.

capacity 255 micrograms per cent). The individual variation in maximum blood levels was wide in the control subjects (Group IV), with a range from 0.66 to 8.8 per cent.

DISCUSSION

The value of the tracer technique in studying iron absorption has been shown by several studies.[11, 18, 19] It seems that the method may be more accurate than earlier chemical iron balance studies where difficulties may arise due to the presence of phosphorus-iron compounds.[2] However, the radioiron method is itself open to misinterpretations, as it is impossible to obtain a complete reflection of iron absorption patterns with only a single dose of labeled iron. In addition Dubach and co-workers[11] recently showed that earlier results in radioiron absorption studies are partly invalidated by their assumption that the quantity of radioiron circulating in the blood was equal to the amount absorbed. Dubach therefore extended the technique to include assessments of the quantities of unabsorbed radioiron present in feces, and was able to show suggestive evidence that normal and abnormal subjects may absorb more iron than they immediately utilize for hemoglobin formation.

In a recent paper[1] we studied the pattern of iron absorption in a case of idiopathic hemochromatosis by blood and feces analyses. In addition to this, in vivo measurements over body organs were also included. By this method we showed a high uptake of radioiron from the gut with significant blood levels and heavy depositions in the liver. Previous absorption studies in this disease have given conflicting results. Chemical iron balance investigations have shown an abnormal retention in only two cases,[12, 23] while no abnormality could be detected in several others.[9, 12] Tracer iron studies have also given conflicting results as Balfour and co-workers[2] could show no evidence of increased iron absorption in a case investigated by them on two occasions, while Dubach and co-workers[11] showed a significant absorption in another. It is possible that the apparently low uptake in Balfour's patient was due to the fact that only blood measurements were done.

In the present study there was a tendency for a greater uptake of iron to occur in idiopathic hemochromatosis as compared with the control and other groups. In two cases (Nos. 1 and No. 3 [2nd dose]) this was strikingly so. It is of interest that in the only two young subjects with idiopathic hemochromatosis investigated by us (Case 1 in the present series and another case previously reported[1]) very high figures for absorption of radioiron were obtained. This finding offers a possible explanation for the poor prognosis in younger subjects.[1] We have suggested previously that the cardiac damage so frequently found in these patients may be due to the insult of the increased rate of deposition of iron in cardiac muscle which occurs secondary to the high intestinal absorption. Blood and in vivo counts in the cases of idiopathic hemochromatosis showed that a proportion of the absorbed iron was utilized for hemoglobin formation and that the remainder was deposited in the liver and other organs. The range of blood uptake in this disease was very similar to that in the control group (1.8

to 6.8 per cent as compared with 0.66 to 8.8 per cent). However, as the excretion of radioiron in feces was less in at least some of the cases of idiopathic hemochromatosis, and as significant organ deposits were present in all cases it seems probable that the actual percentage of absorbed iron utilized for hemoglobin formation was lower than normal. This observation agrees with Finch[13] and co-workers' finding of a low percentage utilization of intravenously administered iron in this disease.

Experimental evidence has suggested that dietary factors may play a part in regulating iron absorption. Increased absorption has been produced in cats after ligation of the pancreatic ducts[27, 28] and in rats fed on an iron-supplemented corn grit diet.[22] This increased absorption could be prevented partially in the cats by the addition of Haliver Oil to the diet,[28] and in the rats by the addition of phosphate.[20] On this basis Granick[16] has suggested that such measures deserve trial in idiopathic hemochromatosis in spite of the lack of evidence of undernutrition as an etiologic feature. Three of our subjects were therefore given various phosphate Haliver Oil mixtures in large doses prior to the administration of a second dose of radioiron, several months after the first one. The results showed no evidence of any blocking of iron absorption. Indeed the blood uptakes and in vivo counts on the whole were higher in the second study. The findings in this second study also seem to show that the rate of iron absorption in idiopathic hemochromatosis varies from time to time in the same individual, although the pattern of high intestinal absorption, heavy organ deposition, and a relatively low hemoglobin utilization is constant.

Results in malnutritional cytosiderosis were conflicting. The average uptake in blood was low. The total uptake of iron from the gut is not known because of difficulties in feces collection. However, it was probably low in at least four of the cases as blood levels were very low and there was no evidence of any significant organ deposits. The remaining subject (Case 9) is of interest, as he was the only one studied while still on the kaffir beer and maize porridge which forms the basis of the average South African Negro's diet. His blood uptake was 2.8 per cent and, he showed a significant deposition of radioiron in the liver (see Fig. 1). Controversy exists as to whether malnutritional cytosiderosis is indeed a disease entity, as recent pathologic studies have shown striking differences between it and idiopathic hemochromatosis. High concentration of iron have been shown in the spleen, while little or no iron has been found in the heart or pancreas.[21] It has been suggested[31] that the high quantities of iron in South African Negro diets (as much as 200 mg. daily[32]) allow for greater absorption, but that this is not necessarily pathologic as Hahn's "mucosal block" has been shown to be only a relative one.[11] However, Case 9 seemed to absorb a significant percentage of the small dose of 20 mg. of radioiron and to deposit a proportion of it in the liver in an abnormal fashion. The low absorption in the other four subjects was possibly related to the fact that they had been on normal hospital diets for some time prior to the study. However, this is pure speculation, and the role of such deficiencies as phosphates, fat-

soluble vitamins, and pyridoxine in influencing iron absorption in this condition requires further tracer studies.

There was, on the whole, a low uptake of radioiron in transfusional hemosiderosis. Feces measurements suggested that about 10 per cent of the dose was retained and only a small proportion of this was utilized for hemoglobin formation in two of the cases. A hemoglobin uptake of 3.5 per cent was present in one subject (Case 12). This finding is of importance in so far as it shows that iron may continue to be absorbed in this condition in spite of enormous body iron stores. It thus offers a possible explanation for the previously unexplained autopsy finding of more iron in the livers of some cases of transfusion hemosiderosis than was present in all the blood transfusions which they had received.[8, 24, 33, 34] In one subject (Case 13) counts above background were obtained over the liver on both the occasions in which she was investigated. This finding suggests that some iron which had been absorbed was being stored in the liver. The presence of anemia did not influence the degree of iron absorption in this case.

The figures for iron absorption and utilization in the control subjects were more or less similar to those found by Dubach and co-workers[11] in normal cases, although their figures for blood uptake were greater than ours, in spite of the fact that they gave ± 3 times the dose which we used. This may have been partly due to the fact that all our cases were suffering from some form of disease (see Table I). Dubach noted a wide individual variation in blood uptake under standard conditions, and this was also a feature of the present study where a range of 0.66 to 8.28 per cent of the administered dose was found in the blood. Suggestive evidence was obtained that some of the radioiron taken up by the body was not being utilized immediately for hemoglobin formation in the four control subjects who had feces analyses done. An average of 18.4 per cent was retained, while only 3.3 per cent appeared in the blood. Although too much significance cannot be attached to these results because of inaccuracies in feces measurements similar findings have been reported by Dubach and co-workers.[11]

SUMMARY

The pattern of radioiron absorption was studied in idiopathic hemochromatosis, malnutritional cytosiderosis, and transfusional hemosiderosis, and the results were compared with a group of control subjects. The passage of the iron was followed by feces analyses (except in malnutritional cytosiderosis), multiple blood sampling, and in vivo measurements.

Increased iron absorption was found in idiopathic hemochromatosis. A proportion of the absorbed iron was utilized for hemoglobin formation, and the remainder was deposited in the liver and other organs. Neither phosphates nor fat-soluble vitamins were found to influence the rate or pattern of absorption.

The absorption of iron from the gut seemed low in four cases of malnutritional cytosiderosis. In the remaining one a significant blood uptake and an abnormal deposition in the liver were found.

Iron absorption was not completely absent in transfusional hemosiderosis, although hemoglobin utilization was very low in all except one subject. It would seem that absorption of iron may continue to occur in this condition in spite of saturated body iron stores.

There was a wide individual variation in the level of radioiron in the hemoglobin of the control subjects and suggestive evidence that all the absorbed iron was not necessarily being immediately utilized for hemoglobin formation.

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MEASUREMENT OF THROMBOPLASTIC FACTORS AND PROFACTORS IN PLASMA

I. Deficits in Thromboplastin. Study of Reagents. Measurements of Antihemophilic and of Platelet Activities

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INTRODUCTION

THE primary difficulty in any study of thromboplastic factors is that of defining the factors being measured. The impossibility of giving a chemical definition (a common occurrence in the domain of coagulation) forces us to adopt a *functional conception*. Our definition will be the following: those factors are thromboplastic which are indispensable and sufficient to form thrombin in the presence of an optimum quantity of calcium and a constant excess of all other known factors of coagulation,† namely, prothrombin, proconvertin (factor VII, cothromboplastin, S.P.C.A.) and proaccelerin (factor V, AC-globulin, labile factor). In these conditions the speed of coagulation of the system will depend only upon the thromboplastic factors, or profactors (platelet factor, antihemophilic factor). If a new coagulation factor were to be discovered tomorrow it would be sufficient to assure ourselves that this factor is also in excess in our reagent, otherwise to add it to the reagent, for the method to keep its value.

The reagent for any method claiming a precise and specific measurement of thromboplastic activity should satisfy the following requirements: (a) Be totally devoid of the factor being measured, thus strictly incoagulable after recalcification at 37° C., even in a glass tube, but coagulate rapidly with traces of thromboplastin. (b) Contain an excess of all the other known factors of coagulation, so that variations in these factors in the plasma being tested be negligible compared with the quantities contained in the reagent. (c) Not be an artificial medium composed of coagulation factors which have been "isolated," since the chemistry of proteins does not at present permit true purification. The products always contain impurities, and the fractionation often partially activates or denatures the factors.

Our purpose in this study is to present two procedures for the evaluation of thromboplastin and profactors. One concerns the measure of antihemophilic activity using human hemophilic plasma together with platelet factor; the other permits the estimation of platelet activity using horse plasma as reagent.

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†Owren terminology is used in this study.

MATERIALS AND METHODS

1. *Description of Reagents.*—We are using two reagents (horse plasma and hemophilic plasma), both incoagulable, which differ in their antihemophilic content.

A. *Horse Plasma Reagent* (free of thromboplastic factors except antihemophilic factor): *Preparation:* Horse blood, taken from the jugular vein on the living animal, is centrifuged for thirty minutes at 2,000 r.p.m. to separate the red cells. The oxalated plasma is centrifuged three times at twenty-four hour intervals for one hour at 4,000 r.p.m. (International refrigerated angle centrifuge) being kept in the centrifuge at a temperature of 1° C. during the intervals. The prolonged centrifugation (three hours' total) completely frees the plasma

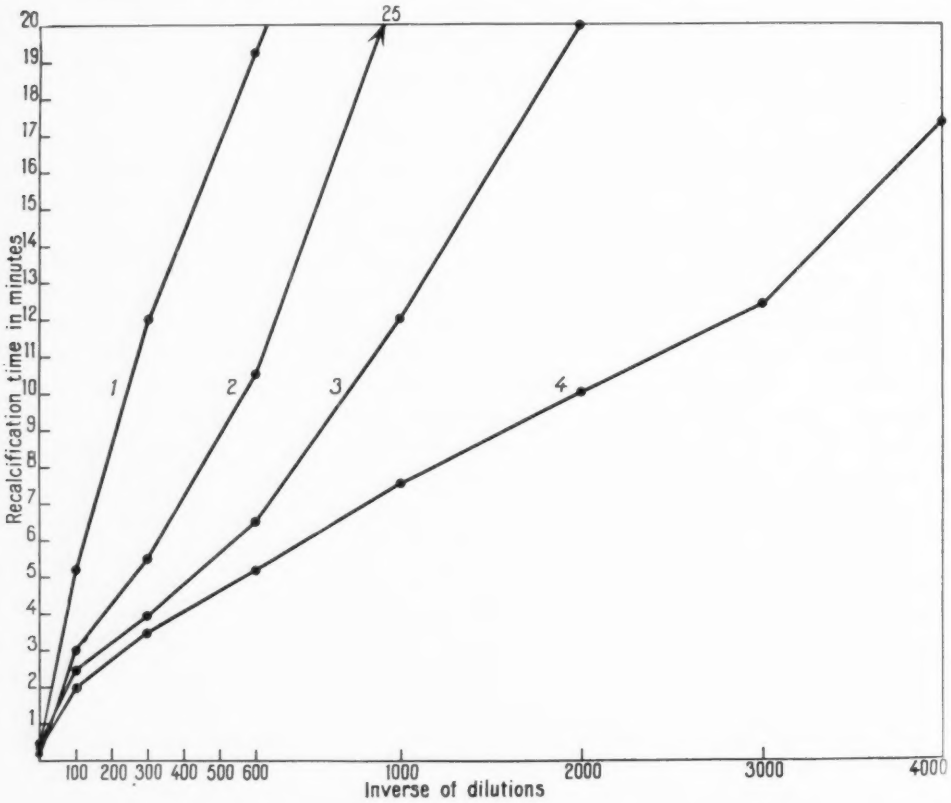

Fig. 1.—Sensitivity to various thromboplastins of horse plasma reagent.
(Thromboplastin suspensions have been heated twenty minutes at 50° C.). (*1*) Old preparation of brain thromboplastin. (*2*) Fresh preparation of brain thromboplastin. (*3*) Geigy thromboplastin. (*4*) Roche thromboplastin.

both of its platelets and of its thromboplastin (whether preformed or formed during coagulation). At +1° C. a precipitate forms of profibrin and thromboplastin (which no doubt plays an important part in carrying down the platelets). The resulting plasma (whether silicone or glass be used) is *strictly incoagulable* with recalcification in glass tubes at 37° C. However, it coagulates very rapidly with thromboplastin: the recalcification time with an equal volume of standard thromboplastin solution is twenty-three seconds and, using the standard solution diluted 1:1,000, the reagent clots in seven minutes (Fig. 1). If very little pro-fibrin forms, it may happen that the plasma remains coagulable upon recalcification. In that case, the following procedures are necessary: Recalcification in the cold with one-fourth plasma volume of $CaCl_2$ at 1.11 Gm. per cent (0.10M). The precipitate of calcium oxalate adsorbs the

residual thromboplastin. Let the mixture settle for twelve hours and centrifuge. The clear supernatant plasma is incoagulable upon recalcification, with a Quick time generally less than twenty seconds. Before freezing the plasma, add one part of sodium citrate (3.8 per cent) to nine parts of plasma.

The reagent contains high proportions of the necessary coagulation factors. Using the techniques of Owren and of Koller[3] we have observed, as is shown in Table I: (a) the progressive prolongation of the recalcification time (Howell time), the plasma after two and one-half days becoming *incoagulable*. (b) The prothrombin time, slightly longer originally than that of a human control (twenty seconds compared with fifteen seconds), becomes only three seconds longer, while the plasma becomes incoagulable. (c) The other clotting factors remain (proconvertin, proaccelerin) as well as the antihemophilic activity. The final fibrinogen level is 4.20 Gm. per 1,000. (d) The antithrombin content is slightly superior to that of a normal human plasma: forty-seven seconds per forty seconds.

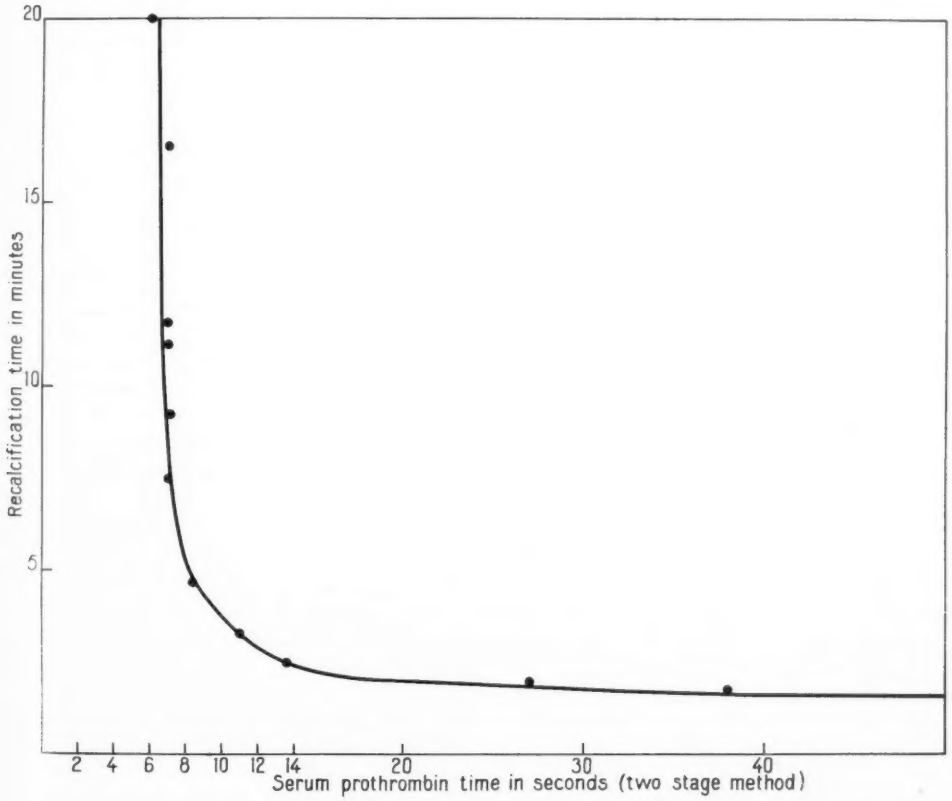

Fig. 2.—Relationship between recalcification time and serum prothrombin time of the horse plasma reagent with serial dilutions of thromboplastin. (1:10 to 1:800.)

Assuming normal human plasma values as 100 per cent for each coagulation factor, we found the following percentages in the horse plasma reagent: thromboplastin 0 per cent, prothrombin 50 per cent, proconvertin 78 per cent, proaccelerin $>$ 100 per cent, fibrinogen $>$ 100 per cent, antihemophilic factor 60 per cent. Because of the 1:20 dilution, the concentration of each factor in the plasma being tested ought to average between 0 and 5 per cent. Therefore the quantitative relationship of the factors in reagent and test plasma is about 10

TABLE I. MODIFICATIONS OF THE COAGULATION FACTORS FOLLOWING CENTRIFUGATION OF OXALATED HORSE PLASMA (1 HOUR CENTRIFUGATION AT 4,000 R.P.M. ON 3 SUCCESSIVE MORNINGS)

RESULTS = REAGENT AT TIME/CONTROL TIME (HUMAN PLASMA)

	5-14-52 AT 10 A.M. AFTER CENTRIFUGATION 20 MIN., 2,000 R.P.M.	5-14-52 12 NOON, AFTER 1ST CENTRIFUGATION 1 HOUR, 4,000 R.P.M.	5-15-52 12 NOON, AFTER 2ND CENTRIFUGATION 1 HOUR, 4,000 R.P.M.	5-16-52 12 NOON, AFTER 3RD CENTRIFUGATION 1 HOUR, 4,000 R.P.M.
Howell time (min.)	4	9.30	31	No clot
Quick time (sec.)	20/15	17/15	26/15	23/15
Proaccelerin (sec.)		20/22	23/22	23.5/24
Prothrombin (sec.)			25/23	27/21
Proconvertin (sec.)			28/23	24/22.5
Antihemophilic factor (min.)			5.15/6	6/5

times more prothrombin, 15 times more proconvertin, 20 times more accelerin, 20 times more fibrinogen, and 12 times more antihemophilic factor in the reagent than in the plasma to be assayed (Fig. 2). This relationship suppresses the effect of any variation of factors other than thromboplastic, giving specificity to the method.

B. *Hemophilic Plasma Reagent* (free of all thromboplastic factors): The only means of obtaining a constant reagent having an excess of all other coagulation factors, except AHF (antihemophilic factor), is from the hemophiliac himself. We have collected on sodium oxalate (final concentration 1.5 Gm. per 1,000) in chilled *siliconed materials* 200 ml. of blood from an adult suffering from typical familial hemophilia. This blood is treated as is the horse blood—primary centrifugation for twenty minutes at 2,000 r.p.m., the decanted plasma is centrifuged for one hour at 4,000 r.p.m. (at 1° C.), the plasma is then left in the centrifuge at 1° C. for twenty-four hours (without decantation) and recentrifuged twice the same day (one hour, 4,000 r.p.m.) before being stored in the freezer at −20° C. This plasma is strictly incoagulable, though it still contains traces of AHF. We suggest distribution of the plasma in 0.1 ml. samples before freezing, thus permitting a notable economy in this precious reagent.

We will not repeat here what has already been said for the horse plasma reagent. All the known factors of coagulation are in excess compared to their quantities in the plasma to be assayed (diluted 1:20) except the antihemophilic factor.

C. *Platelet Reagent:* It is an *extract of human platelets*, deprived of the antihemophilic plasma factor by heating.

Five hundred milliliters of fresh citrated human blood was centrifuged for five minutes at 1,800 r.p.m. The turbid plasma was pipetted and recentrifuged for five minutes at 180 r.p.m. to separate the residual erythrocytes and leukocytes. It was then possible to pipette 180 ml. of plasma rich in platelets (180,000 per mm.^{3}) which was then centrifuged for one hour at 1° C. at 4,000 r.p.m. The compact sediment of platelets obtained was taken up in 180 ml. of distilled water, put into suspension, and heated for thirty minutes at 64° C. (three minutes at 64° C. are sufficient to destroy completely the antihemophilic activity, thirty minutes destroys in addition a large part of the semithermolabile thromboplastic lipoprotein, leaving a thermostable platelet factor). To insure a finer and more homogenous suspension, the mixture is stirred violently with a stirring rod for fifteen minutes, then frozen and defrosted twice, this process facilitating the complete disintegration of platelets.[13, 33] This final suspension, centrifuged for five minutes at 2,000 r.p.m., has been used in our experiments under the name of *platelet factor*.

2. *Other Requirements.*—

Calcium optimum: $CaCl_2$ solution $\frac{M}{40.}$

Temperature: The test is made at 37° C.: The reagents are kept frozen at −20° C. until ready for use. Once brought to room temperature, they would, on standing three hours,

increase slightly the reaction time (from 7 minutes to 9 minutes), whether kept in glass or in silicone. One the contrary, plasma to be tested, on standing three hours at room temperature, would decrease the reaction time only if diluted. When undiluted it remains constant.

Contact.—

(1) *Blood Collection and Contact:* We found a standard technique of collection providing a *constant* contact more advantageous than a complicated technique using silicone-coated surfaces. Therefore, the technique for blood collection of the plasma to be tested is the same as the one we use for the prothrombin and the "in vitro" heparin tolerance tests.[15] This procedure allows these three tests to be done on the same blood sample. Ten milliliters of blood are drawn by direct venipuncture and collected in a flask containing 1 ml. of Wintrobe[1] solution. The flask is inverted two or three times, centrifuged at 1,800 r.p.m. for five minutes under the same conditions as a control specimen. If the plasma is not tested at once, it has to be stored, as the control, in the refrigerator. The 1:20 dilution is made only previous to testing. In order to obtain a good clot, one has to dilute the plasma to be tested with a buffer at pH 7.3. We use a veronal-acetate—HCl buffer, according to Owren's technique.

(2) *Reaction and Contact:* The reaction times differ completely depending on whether the test is done in glass or in siliconed tubes. Times are found much longer in silicone. Since shorter times are preferable, the test is made in duplicate in *glass* tubes, tilting the tube every thirty seconds.

Special procedures for each of the two tests will be given preceding their clinical applications.

RESULTS

I. *Specific Measurement of Antihemophilic Activity: Hemophilia.*—

Procedure: When to the incoagulable hemophilic reagent (HR), a constant excess of platelet factor (PF) and the plasma diluted 1:20 are added, the only variable is the content of antihemophilic factor in the plasma being tested. Four reactions are carried out simultaneously:

(1) 0.1 ml. HR + 0.1 ml. saline + 0.1 ml. $CaCl_2$

(2) 0.1 ml. HR + 0.1 ml. PF + 0.1 ml. saline + 0.1 ml. $CaCl_2$

TABLE II. STUDY OF 14 CASES OF TYPICAL HEMOPHILIA (WHETHER FAMILIAL OR NOT). MEASUREMENT OF ANTIHEMOPHILIC FACTOR (AHF) BY COMPARING $\frac{\text{PATIENT TIME}}{\text{CONTROL TIME}}$

NO.	NAME	AGE IN YEARS	FAMILIAL OR SPORADIC	CLOTTING TIME (IN MIN.)	SERUM PROTHROMBIN TIME	AHF PATIENT CONTROL	HR + PF	PERCENTAGE OF AHF (NORMAL BEING 100 PER 100)
1	T. R.	5	F		6 sec.	9.45/3.30	9.45	0
2	L. E. C.	$2\frac{1}{2}$	S	22	5 sec.	9/4.15		5
3	P. O.	1	F	8–9	5 sec.	6.15/4.15	9	21
4	D. I.	45	F	9–12	9 sec.	7.45/3.45	11	5
5	B. R.	10		95	5 sec.	7/3.15	7	0
6	A. U.	9	F	60–80	5 sec.	8/4.15		7
7	B. E.	7	S	27	14 sec.	6.15/3.45	7.30	15
8	H. E.	45	F	65	9 sec.	6/3.45	9	18
9	C. H.	35	F		10 sec.	9.15/3.45	9	0
10	D. E.	7	S	60	7 sec.	8.15/4.30	9.30	8
11	V. A.	10	F	80	6 sec.	7.30/2.45	7.30	0
12	Le C. O. R.	11	?	45	8 sec.	8/3.30		2
12 bis	Le C. O. R. 23/6	11	?		8 sec.	6.30/2.15 10.15/4		2 1
13	Le C. O.	16	F	11–14	12 sec.	déb. 8	déb. 8	0
14	G. O.	18	F	240	5 sec.	fin 15 3	fin 15	

(3) 0.1 ml. HR + 0.1 ml. PF + $\frac{0.1 \text{ ml. control plasma}}{20}$ + 0.1 ml. $CaCl_2$

(4) 0.1 ml. HR + 0.1 ml. PF + $\frac{0.1 \text{ ml. patient plasma}}{20}$ + 0.1 ml. $CaCl_2$

Sample results in hemophilia:

Saline control	(1) :	∞
PF control	(2) :	11 minutes, 30 seconds
Normal plasma + PF, control	(3) :	4 minutes
Experimental	(4) :	11 minutes

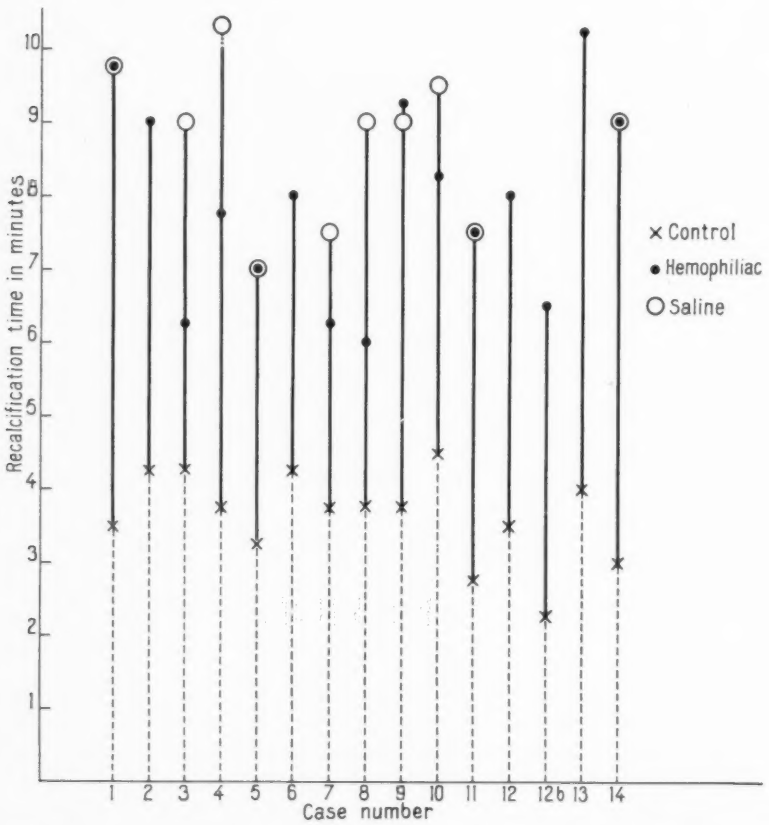

Fig. 3.—Recalcification time using hemophilic and platelet reagents. For each case 3 points are given on a vertical : control, patient, and saline.

Diagnosis of hemophilia: We have tested *14 hemophiliacs* (one of them twice) and obtained in every case constant and clear-cut results leaving no diagnostic doubt (Table II). The deficit in antihemophilic factor shows up distinctly with this method, *even when the coagulation time of venous blood is normal* and even when the prothrombin consumption is only moderately disturbed (Cases 3, 4, 7, and 13). *The specificity* of the method is superior to that of all others, since the prothrombin consumption is disturbed not only in hemo-

philia but also in thrombocytopenia as we have shown in 1948,[34a] a fact confirmed by Quick, Brinkhous, and other authors.

Fig. 3 is a diagram showing the three different times obtained in the presence of platelet factor (patient plasma, control plasma, and saline). The *difference* between patient and control times is directly related to the patients'

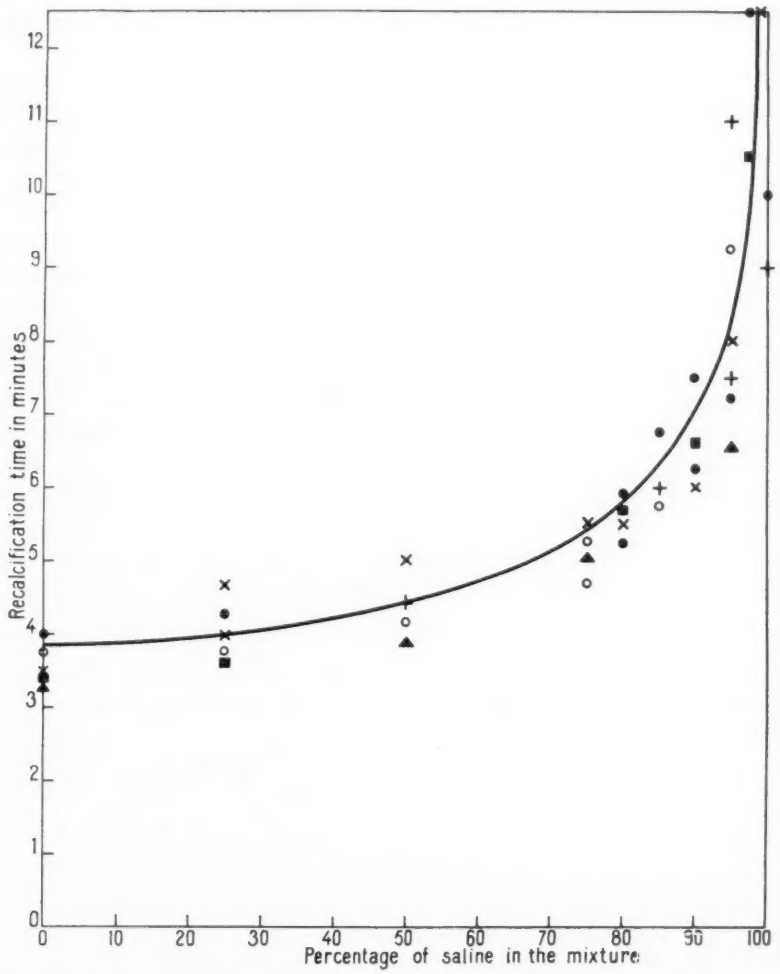

Fig. 4.—Effect of dilution with saline of a control plasma (already diluted 1:20), adding a constant amount of platelet factor.

lack of AHF. If the plasma is practically without AHF, the time obtained coincides with that obtained with saline. For determining the percentage of the AHF we established a dilution curve upon mixing a control plasma (diluted 1:20) with increasing proportions of saline from 0 to 100 per cent).

Fig. 4 shows the dispersion along an ideal curve in successive experiments during several weeks. If the time found for the tested patient is plotted on a

theoretical curve, starting on the ordinate at the time given by the control and parallel to this ideal curve, one can read on the abscissa the percentage of AHF accurate to about 5 per cent.

Example: Case 12, tested twice:

9-6-52—8 minutes/3 minutes 30 seconds = approximately 2 per cent
23-6-52—6 minutes 30 seconds/2 minutes 15 seconds = approximately 2 per cent

In considering the values shown in Table II, one is struck by the extremely low level of antihemophilic factor possessed by the hemophiliac, usually less than 10 per cent of the normal. This is true even when the coagulation time is normal or only slightly prolonged (Cases 4 and 13). The highest percentage we have found, that of Case 3, is 21 per cent of normal. In this case, an infant of 11 months, it is possible that, due to the difficulties of venipunctures, the sample was contaminated with tissue thromboplastin.

In five cases the plasma of the patient gave the same results as the saline solution, indicating a level of antihemophilic factor practically 0 (Cases 1, 5, 9, 11, 14, see Fig. 3). Note also, in Fig. 3, that in spite of individual variations the times given by all hemophiliacs (even the shortest time) are above five minutes, while all the controls' times (even the longest) are below five minutes. Finally let us point out that the difference between the hemophiliac and the control is undoubtedly due to the antihemophilic factor, since heating three minutes at 64° C., which destroys this factor, annuls the difference between the times for the control, the hemophiliac, and the saline.

II. *Measurement of Deficits in Platelet Activity: Thrombocytopenias.*—

Procedure: The horse plasma reagent, being devoid of platelets and of free thromboplastin is very sensitive to thrombocytopenia and in a more general way to hypoprothromboplastinemia. The reaction is as follows:

(1) 0.1 ml. horse plasma reagent + $\frac{\text{0.1 ml. plasma to be tested}}{20}$ + 0.1 ml. $CaCl_2$

Compared with:

(2) 0.1 ml. horse plasma + $\frac{\text{0.1 ml. control plasma}}{20}$ + 0.1 ml. $CaCl_2$

(3) 0.1 ml. horse plasma + 0.1 ml. saline + 0.1 ml. $CaCl_2$

Sample results in thrombocytopenia:

Experimental	(1)	20 minutes
Normal plasma control	(2)	7 minutes 30 seconds
Saline control	(3)	∞

In every case of thrombocytopenia the time of the patient is clearly longer than of the control, no matter what the cause of the thrombocytopenia may be: idiopathic thrombocytopenia, acute leukemia, bone marrow aplasia.

This difference between control and patient plasma increases if the plasma dilution of 1:20 is centrifuged ten minutes at 3,800 r.p.m. and the upper super-

natant is tested. No clot results with the pathologic plasma, while with the control plasma, clotting occurs in fifteen to forty-five minutes.

Table III shows nine cases of thrombocytopenia studied. In two cases, plasma was tested after the patient's recovery. In Case 9B the reaction time returned to normal. Case 10 still shows a small deficit in platelet factor which agrees with the number of platelets found (148,000). This test, although it is not specific for thrombocytopenias, gives constant and sensitive results. It may prove to become a valuable test for thrombopathies without thrombocytopenia. We could test only three cases of Willebrand's disease exhibiting delayed bleeding time and normal platelet counts. All three plasmas showed normal thromboplastic activity.

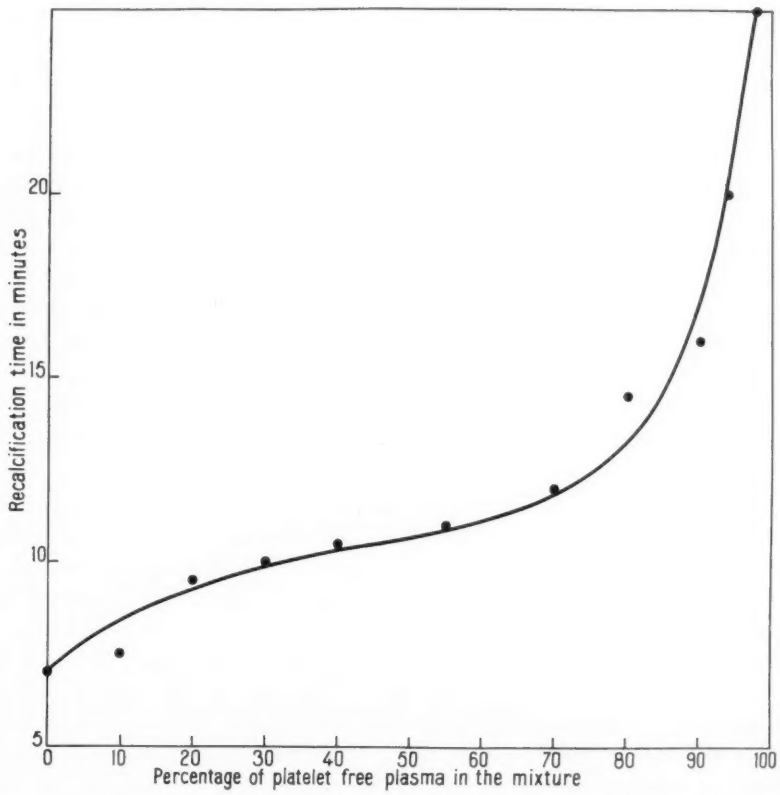

Fig. 5.—Artificial thrombocytopenia in vitro by dilution of control plasma (already diluted 1:20) with the same plasma made platelet-free by centrifugation.

Study of artificial thrombocytopenias in vitro. (Dilution's curves): In order to study quantitatively the role of the platelets, we have made a series of dilutions by mixing a 1:20 dilution of normal plasma with increasing amounts of the same plasma dilution centrifuged one hour at 4,000 r.p.m. to remove the platelets. We have obtained a series of recalcification times, between seven and twenty-three minutes in one experiment, and between seven

TABLE III. STUDY OF TEN CASES OF THROMBOCYTOPENIA. MEASUREMENT OF PLATELET ACTIVITY BY COMPARING PATIENT/CONTROL TIME, WITH 1:20 DILUTED PLASMA NOT CENTRIFUGED AND CENTRIFUGED. (CONTROL TIME FOR CENTRIFUGED PLASMA VARIES BETWEEN TWENTY AND FORTY-FIVE MINUTES)

NO.	NAME	PLATELET	CLOT RETRACTION	PLASMA 1:20 NOT CENTRIFUGED (TIME IN MINUTES)	PLASMA 1:20 CENTRIFUGED (TIME IN MINUTES)
1	B. E.	37,000	0	19/8	60+
2	D. E.	52,000	0	12.30/8.15	60+
3	L. A.	20,000	0	14/6	60+
4	J. O.	18,000	0	22/6.15	60+
5	T. I.	80,000	±	24/16.45	60+
6	R. E.	14,800	0	25/7.30	60+
7	Boul.	60,000	0	20/7.30	?
8	Bouv.	45,000	0	21/10	60+
	D. A.	14,000		38/6.15	60+
9 bis	D. A.	230,000	+++	10.30/9	21
10	P. E.	148,000	+++	15/8.30	34

and twenty-six minutes in another. The curve obtained permits an approximate calibration of the platelet activity (Fig. 5) and a comparison with the curve of total thromboplastic activity (Fig. 6) obtained when saline is used as diluent, instead of centrifuged diluted plasma. This comparison shows that the coagulation effect of the tested plasma on the reagent is due essentially to the platelets. When they are deficient, the variations in free thromboplastin (preformed, or formed at the moment of collection) play only a small and accessory role compared with variations in platelets. It is not the same when an *excess* of thromboplastic factors is measured, a problem which we do not plan to include in this study.

Estimation of over-all deficit in thromboplastic factors: Both reagents (hemophilic plasma and horse plasma) allow estimation of the total thromboplastic activity of a plasma.

In hemophilia, as in thrombocytopenia, where one of the profactors necessary for the formation of thromboplastin is lacking, the reaction with the plasma to be tested diluted 1:20 is longer than that of the control. Examples: 18 minutes, 11 minutes, 15 minutes, 35 minutes, 15 minutes 30 seconds for a control of 6 minutes 15 seconds.

This is an important argument for the view that the traces of active thromboplastin found in normal plasma are formed at the moment of collection of the blood, before decalcification by the oxalate. Free thromboplastin is not found in blood collected from a hemophiliac or in thrombocytopenia, since the deficit of one of the two profactors of thromboplastin prevents its endogenous formation during the collection of blood.

DISCUSSION

In the interest of clarity we divide the discussion into three parts: (1) brief presentation of the concepts and terminology of thromboplastic factors in the plasma; (2) discussion of the choice of the horse plasma reagent as "stable plasma"; (3) discussion of the attempts at quantitative measurement of thromboplastic factors and profactors.

1. *Terminology and Concepts Concerning Thromboplastic Activity of Plasma.*—To interpret our findings, it is necessary briefly to consider the current concepts found in the literature about thromboplastin. Opinion differs as to whether the active thromboplastin originates in the plasma, the platelets, or in both.

(a) *Platelet factor:* Rare are the authors who, like Lengenhagger,[11] deny any role to the platelets. Most authors admit the old concept of Bordet, of a thermostable lipoid (cytozyme) which acts with a plasmatic profactor. The platelets are poor in thromboplastin (Copley and Houlihan, Quick, Ware,

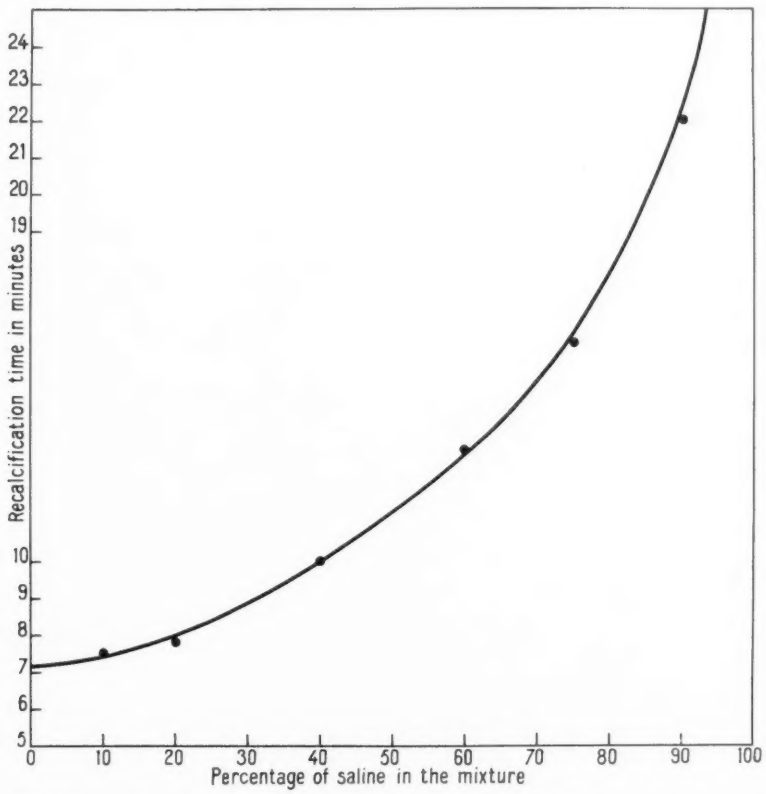

Fig. 6.—Dilution of thromboplastic factors by dilution of control plasma (already diluted 1:20) with saline.

Fahey, and Seegers) and may contain other accessory factors (Van Creveld[35]). Brinkhous[10] postulates for platelet disintegration the activity of a plasma thrombocytolysin.

(b) *The thromboplastic plasma factor or factors:* We have found 4 different concepts: (1) *Profactor* of a thrombokinase or of a plasma thromboplastin. Lengenhagger[11] followed by Schmid,[12] Dale and Walpole, followed by Milstone,[21] describe a preformed plasma thrombokinase in the form of an inactive precursor, prothrombokinase. This protein is activated by thrombo-

catalysin according to Lengenhagger and Schmid, and by surfaces and calcium alone according to Milstone. (2) *Plasma thrombokinase:* Others do not speak of a prothrombokinase, but only of a *thrombokinase, or plasma thromboplastin* (Howell), which precipitates at low temperatures (Owren[24]), is retained by Chamberland or Berkefeld filters (Cramer and Pringle,[5] Clowes[26] and Eagle[6]), is adsorbed by tricalcium phosphate, aluminum hydroxyde, and kaolin (Lenggenhager), or by calcium oxalate (Nolf and Adant[16]). (3) *Proteolytic substance in the plasma:* Fredericq[23] and Ferguson and associates[36] do not speak of thrombokinase but of *tryptase or protease,* which they compare to plasma protease. This tryptase is inactive in the circulating blood (pro-tryptase). (4) *Antihemophilic factor:* After Govaerts and Gratia,[33] Bendien and Van Creveld,[19] the Thorndike group at Boston[17] places the accent on an antihemophilic factor, Conley and co-workers[32] call it plasma thromboplastin precursor. Quick names it thromboplastinogen.

From the studies mentioned above and from our own work we may conclude as follows: an *antihemophiliac plasma factor* and a *platelet factor* exist, both indispensable. When one of them is lacking, no prothrombin is consumed. These two factors appear to react with each other (though one cannot say which is the substrate), in the presence of contact and calcium, to form an *endogenous neothromboplastin.*

Besides these two profactors there is in plasma collected in the usual way a thromboplastin (or active thrombokinase) which can be eliminated by Berkefeld filtration, or by prolonged centrifugation at 31,000 g or by cold centrifugation (all are operations which leave antihemophilic factor intact). It is possible that this plasma thromboplastin is normally preformed in the plasma, but we rather believe that it appears as the result of two possible conditions: one is physiologic, resulting from the collection of the blood (endogenous neoformation of thromboplastin can take place before decalcification, not speaking of accidental contamination with tissue juice); the other is pathologic, such as in certain states of hypercoagulability (which we plan to discuss in another publication). In the normal state the circulating blood does not appear to contain active thromboplastin.

2. *Choice of the Oxalated Horse Plasma as Stable Plasma for Thromboplastin Assays.*—Before adopting horse plasma reagent, we made numerous attempts to find the most suitable stable plasma—calling *stable* a plasma not clotting on recalcification.

(1) The cold centrifugation at 4,000 r.p.m. is effective only when using *horse oxalated* plasma. It is not possible to obtain an incoagulable plasma when using *citrated* horse plasma or *human* and *beef* oxalated plasmas. Brinkhous[19] needed to obtain an incoagulable *human* plasma twenty hours of cold centrifugation, and Chargaff[9] used high speed centrifugation. In both procedures participate not only the platelets but also the large molecules of thromboplastin.

(2) *Other stable plasmas that we tried:* We found centrifuged *goose* plasma (as used by Fischer) only slightly sensitive to human thromboplastin. We prepared *superoxalated plasma* according to Nolf.[4] This plasma, whether

horse, human, beef, or goose plasma, is incoagulable but due to the dilution (necessary to re-establish a normal ionic concentration) is sensitive to clotting factors other than thromboplastin. Numerous attempts to obtain an incoagulable plasma by filtration through *Berkefeld filter* (N and W) did not allow us to get a constant reagent. We were unable to adsorb plasma thromboplastin with freshly prepared tricalcium phosphate added to plasma citrated at 0.2M (Lengenhagger[11] and Schmid[12]).

3. *Discussion of Methods Published for the Estimation of Thromboplastic Factors.*—

(a) *Measurement of "thrombokinase":* Certain authors, such as Meunier and Croizat and collaborators have described hypoprothromboplastinemias based on the aspect of the opacity curve of the recalcified plasma. However, these electrophotometric studies are not more specific of thromboplastin than the classical clotting time. Schmid[12] published the following method: the plasma to be tested is diluted with a plasma made prothrombokinase-free through adsorption by tricalcium-phosphate. The recalcification time is measured, after the addition of thrombocatalysin (trypsin). Besides the fact that the use of trypsin is not physiologically sound, great variations in prothrombin or fibrinogen modify the times observed. It is to be noted that the antihemophilic factor is not measured by this method (Schmid finds in hemophilia an increased level of prothrombokinase).

De Vries and Shafrir[14] have just proposed a method "for estimation of the thromboplastic activity of "plasma," in which the principle is the activation by contact of a plasma deprived of platelets and prothrombin. The clotting activity obtained is measured with a platelet-free plasma. On merely reading this study, one can make the following remarks: the plasma to be tested and the reagent are not diluted, so that any variation of a factor other than thromboplastin also influences the time of coagulation. The reagent contains the factor to be measured (it is not incoagulable). The removal of platelets is far from being complete, and for us is responsible for the clotting activity of the plasma shaken with glass powder. We have carried out the same experiment as de Vries with the horse plasma reagent which contains antihemophilic factor but is totally free of platelets. This reagent shaken with glass powder remains incoagulable after recalcification, and even becomes less sensitive to thromboplastic factors.

(b) *Measurement of antihemophilic factor:* Alexander and Landwehr[31] proposed a method for the estimation of antihemophilic factor, in which the plasma (or the plasma fractions) to be tested are diluted in series and tested with 2 ml. of fresh hemophilic blood. There is a linear relation between the logarithms of the concentration of the plasma tested and those of the clotting times obtained. This interesting relationship makes it possible to compare different plasmas (each hemophilic plasma giving a straight line with its own slope) and to appreciate the corrective properties of plasma or fractions. This method is limited only by the necessity of having fresh hemophilic blood and the fact that the plasma to be tested may form during collection and

manipulation thromboplastin which, at the high dilutions used, adds its effect to that of the antihemophilic factor.

Quick,[2] in his recent monograph, describes under name of test No. 13 an "assay of thromboplastinogen." He uses, as does Alexander, fresh hemophilic blood to which he adds in different proportions the plasmas to be tested. He then measures the prothrombin consumed. This technique *lacks sensitivity*, since in reviewing Quick's results (Table VI) it is seen that the correction of the prothrombin consumption is only four to five seconds for 1:10 dilutions and two to three seconds for 1:20 dilutions, while differences between two normal bloods may range from thirteen to twenty-one seconds.

Brinkhous[10] proposes a very interesting assay of the antihemophilic factor, based on the prothrombin consumption of hemophilic dog blood. But, besides the fact that not everyone has access to a hemophilic dog, his method (even transposed to human hemophilic blood) is complicated and the dialysis and numerous dilutions of the plasma being tested may favor the formation of thromboplastin.

We believe that the method we propose escapes these objections. (1) *It is specific*, since the action of the platelets is eliminated not by the often imperfect method of centrifugation, but by addition of an excess of platelet factor, heated to destroy the AHF. (2) *It is sensitive*. We have abandoned the measurement of consumption of prothrombin, though we were among the first to study and recommend it in the study of hemophilia.[3,4] The measurement of serum prothrombin is very sensitive when the recalcification times are short. Where the coagulation times are long, as with our reagent, serum prothrombin times fall within a very small range and lose their utility. This is well shown in the curve we have established (Fig. 2), using varying dilutions of thromboplastin from 1:10 to 1:800 with the horse plasma as reagent. There is a very sensitive range of serum prothrombin times with recalcification times less than three minutes. On the contrary with longer recalcification times (from five minutes to incoagulability), the serum prothrombin times, using our two-stage method,[3,4b] are very close and difficult to distinguish, varying by only one to four seconds (as in Quick's one-stage technique). Adding that the recalcification time is very simple and not exposed to the errors which arise in the manipulations necessary for a serum prothrombin measurement (often responsible for great differences between controls), it is understandable that we have chosen the more sensitive recalcification time.

SUMMARY

The method is given for the preparation of two reagents which are incoagulable and very sensitive to thromboplastin.

The first reagent (hemophilic) contains neither thromboplastin nor its profactors. The second reagent (horse plasma) does not contain thromboplastin, but does contain antihemophilic factor.

A human platelet factor deprived of antihemophilic factor, by heating thirty minutes at 64° C., is prepared.

The plasma to be tested is diluted 1:20 so that by comparison the reagents contain a large excess of coagulation factors other than the thromboplastic factors.

The plasma diluted 1:20, when tested with platelet factor and hemophilic reagent, gives a quantitative and specific measurement of antihemophilic factor. Twelve cases of hemophilia have been thus studied. The scale established shows that the AHF level in most hemophiliacs is less than 5 per cent of normal.

The plasma diluted 1:20 tested with the horse plasma reagent allows an assay of platelet activity (in the case of thromboplastin deficit). Nine cases of thrombocytopenia illustrate this view.

The evaluation of our finding necessitates a discussion of the principal concepts and terminology of thromboplastin as well as a critical appraisal of of reagents and methods concerned with thromboplastic factors.

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THE EFFECT OF PROLONGED ADMINISTRATION OF EPINEPHRINE ON ADRENAL CORTICAL FUNCTION AND EPINEPHRINE TOLERANCE IN CHRONIC ASTHMATICS

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EPINEPHRINE is probably the most important single substance used in the treatment of bronchial asthma, its importance presumably being due to its sympathomimetic bronchodilator action. This direct action has been considered adequate to explain the beneficial antiasthmatic effect. But, this may not be the whole story since we now know that epinephrine may have a broader, endocrine influence, i.e., the stimulation of adrenal cortical activity via the hypothalamus-pituitary-adrenal pathway. It is established that ACTH and cortisone usually relieve the symptoms of bronchial asthma. Therefore, if the repeated administration of epinephrine resulted in the production of endogenous ACTH and adrenal steroids at beneficial levels, one should expect prolonged relief from asthma. This desirable sequence of events apparently does not occur, since the bronchodilator effect of epinephrine is prompt but brief, while the antiasthmatic effect of ACTH or cortisone is delayed but prolonged.

Patients with bronchial asthma vary in their bronchodilator requirements, depending on the frequency and severity of their attacks, and on their clinical response to the drugs being used. We have had under observation a group of asthmatics who have depended on epinephrine for relief and have taken the drug by injection one or more times daily for months or years. The effects of long-continued epinephrine administration in the human being should be manifest in such individuals. We planned our study in an effort to ascertain the following: (a) Is the hypothalamus-pituitary-adrenal system continually stimulated to activity by epinephrine or does it become refractory at some stage? (b) If the latter should be the case, does temporary discontinuation of epinephrine administration restore the sensitivity of the system?

This group of patients affords the further opportunity for the observation of possible tolerance phenomena to epinephrine taken as stated above. Over forty years ago Abderhalden[1] demonstrated that tolerance to epinephrine does occur. This concept is now generally accepted, but, as stated by Vigran and Essex,[2] the "Nature of tolerance developed to epinephrine still remains an enigma." Barach and Garthwaite[3] in grouping epinephrine, ephedrine, aminophylline, and neosynephrine, described a loss of clinical response after the repeated administration of these bronchodilators, with rapid restoration of response after the substitution of demerol for a few days. Since this may not be an example of tolerance to the drugs but rather a manifestation of increased

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severity of the pathologic state, the study was extended to consider whether the temporary discontinuation of the drug in epinephrine habitués, not in an aggravated asthmatic state, leads to a greater antiasthmatic effect when the drug is again given.

METHOD

The subjects were five men ranging in age from 57 to 67 years, all chronic asthmatics, with varying degrees of superimposed pulmonary pathology, who had taken clinical doses of epinephrine subcutaneously two or more times daily for prolonged periods. During the observation period, except as noted in No. 4 and No. 6 below, the patients received injections (0.3 ml. to 0.5 ml.) of epinephrine (1:1,000) morning and evening and additionally as indicated for asthmatic attacks. Daily sweat tests and twice daily eosinophil counts were done, the latter being made before and four hours after the morning dose of epinephrine. The observation period was divided as follows: (a) daily epinephrine one week (control); (b) 25 mg. ACTH test, one day; (c) daily epinephrine 1 week (control); (d) demerol—50 mg. every six hours; no epinephrine, three days; (e) daily epinephrine, 1 week; (f) ACTH —40 to 80 mg. daily, no epinephrine, ten days.

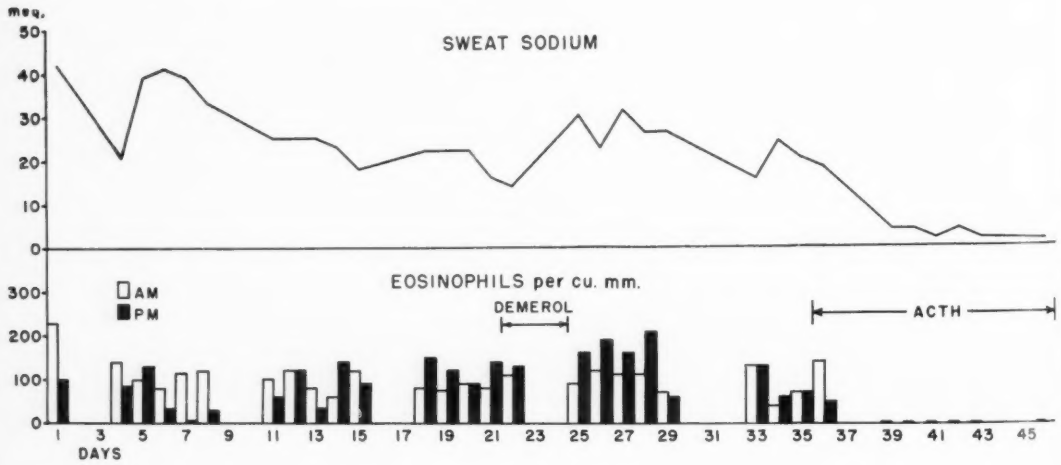

Fig. 1.—Case G. L.

All of the patients were hospitalized for the period of observation, but were ambulatory and on regular diet.

Sweat tests were performed according to the technique of Leslie and Levin.[4] The Beckman flame photometer was used for the sweat electrolyte determinations. Eosinophil counts were done according to the technique of Thorn and co-workers.[5]

RESULTS

During the control periods when epinephrine was administered daily there were so significant or consistent variations in circulating eosinophils. When epinephrine was reinstituted following the three-day period during which demerol was substituted for it, there was still no eosinopenic effect. The significance of this unchanged response will be considered later. The sodium content of the sweat did not change significantly in relationship to the discontinuous administration of epinephrine (Table I; Figs. 1 to 3).

On the other hand, ACTH administration resulted in uniformly significant changes in the sweat sodium. In four of the five patients, this was also true for the level of circulating eosinophils. After ten days of ACTH the sweat sodium dropped from levels well in the normal range to the low average figure of 11 meq. per liter, and the level of circulating eosinophils similarly dropped from normal levels to an average of 28 per cubic millimeter. The four-hour

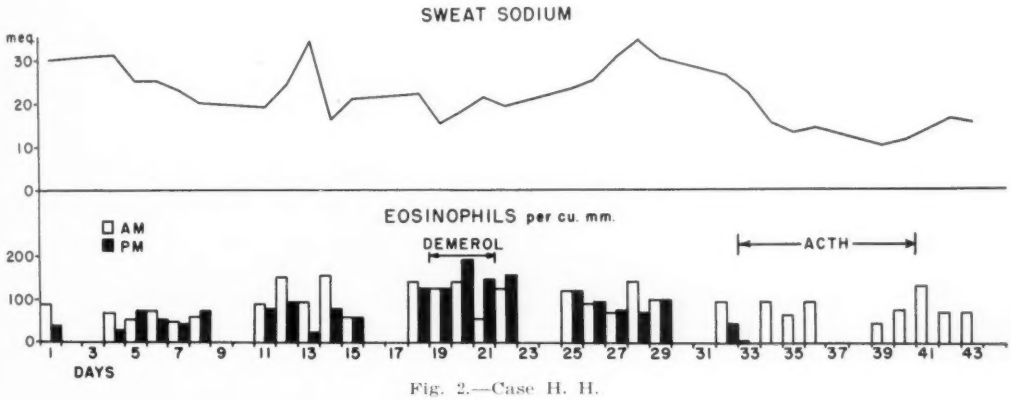

Fig. 2.—Case H. H.

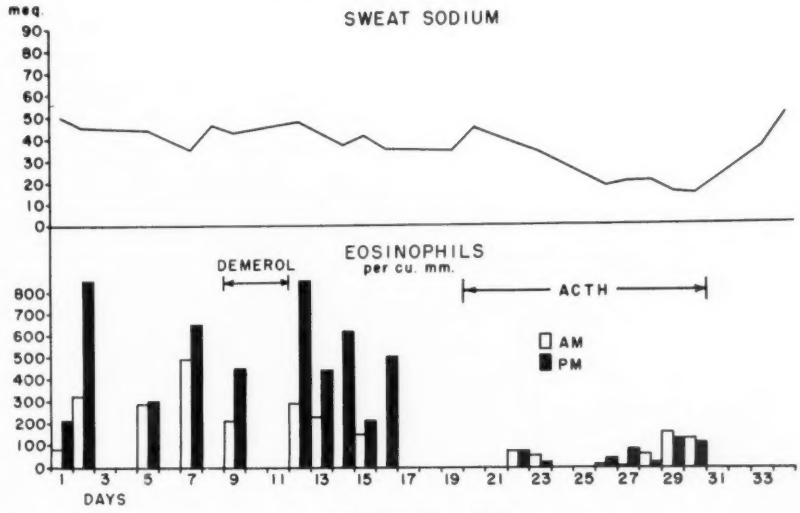

Fig. 3.—Case G. B.

ACTH test[5] done on four patients during the control period produced a significant depression of circulating eosinophils in three (Table I). In the other patient (G. L., Fig. 1) the count dropped somewhat, but not to the extent that would be expected in a subject with a normally responsive adrenal cortex. After ten days of ACTH, however, his eosinophil count dropped from normal levels to zero, presumably indicating responsiveness of the adrenal cortex. Patient H. H. (Fig. 2) who showed a normal drop in circulating eosinophils on the

TABLE I

	CIRCULATING EOSINOPHILS/CU. MM.*		SWEAT Na (MEQ./L.)			ACTH TEST: CIRCULATING EOSINOPHILS/CU. MM.	
	BEFORE EPINEPHRINE	4 HR. AFTER EPINEPHRINE	CONTROL EPINEPHRINE†	NO EPINEPHRINE‡	RESTORED EPINEPHRINE†	BEFORE ACTH	AFTER ACTH
1. G. B.	374	495	61	52	47	--	--
2. G. L.	119	75	33	27	24	130	90
3. G. H.	130	131	20	16	13	200	100
4. H. H.	84	61	25	16	28	94	25
5. R. W.	141	160	35	33	24	188	63
Average	170	184	35	29	27	153	70

*Average of seven observations during control period.
†Average of four observations.
‡Average of three observations.

four-hour ACTH test failed to show the marked eosinopenia which occurred in the other cases after ten days of ACTH therapy.

From the purely clinical point of view, several observations are of interest. None of the patients at any time noted a loss of responsiveness to epinephrine; all reported that definite improvement in ease of breathing or relief of asthma occurred after every injection. Furthermore, no patient noticed the development of tolerance to the drug, with consequently increased requirements for relief. When demerol was administered, in 50 mg. doses at six-hour intervals for three days, the patients' symptoms were also satisfactorily controlled. In fact, no asthmatic attacks occurred during this period, and breathing was relatively easy. At the end of this period, when the patients returned to the epinephrine schedule, their symptoms were essentially the same as before the administration of demerol. None of the patients noted any particular augmentation of epinephrine effect as compared with the pre-demerol period.

Three of the patients noted complete amelioration of asthma after receiving ACTH. Relief occurred within two days of starting the ACTH, and lasted about two weeks after the drug was discontinued. The other two patients, despite a doubled ACTH dosage, when no response was observed after 40 mg. per day, failed to show any improvement whatever. One of these was patient H. H., who showed no eosinopenia after the ten-day course of ACTH.

DISCUSSION

According to some investigators,[6, 7] adrenal function and response to ACTH are impaired in intrinsic asthmatics. The results of our studies in this small group of intrinsic asthmatics are not necessarily at variance with those of these investigators, since different and perhaps less direct observations were made. Nevertheless, we observed no evidence of impaired "salt-activity." Sweat sodium levels were normal throughout the control periods and dropped normally after ACTH administration. Similarly, there was significant eosinopenia after ACTH. Thus, by these criteria there was no abnormality of adrenal response to ACTH.

The role of epinephrine in the elicitation of adrenal cortical secretion is still controversial, opinion being divided as to whether it is an essential link in

the chain of events,[8-10] or merely one of a group of nonspecific stimuli to hypothalamic-pituitary-adrenocortical activity.[11-14] Recant and associates[15] demonstrated quite conclusively that the eosinopenia following epinephrine, which was observed as far back as 1910,[16] is mediated via the pituitary-adrenocortical pathway, and provides a test for the integrity of this pathway. They noted, however, that the mechanism responsible for this eosinopenia rapidly becomes relatively refractory, not only after epinephrine, but also after ACTH. The work of these various investigators indicates that epinephrine, while not the only provocative agent, activates a hypothalamus-pituitary-adrenal mechanism with resulting adrenal cortical activity. This activity, however, is brief, the mechanism rapidly becoming refractory to the further administration of epinephrine. Our findings fit into this general hypothesis. A hypothalamus-pituitary-adrenal cortex response to epinephrine was not evident in these patients who had received epinephrine for prolonged periods. The adrenal cortex had not been stimulated to hyperactivity but responded about normally to administered ACTH (salt activity and eosinopenia).

Segal and Herschfus[17] were unable to demonstrate protection against bronchoconstriction and dyspnea following intravenous histamine or methacholine from large single doses of ACTH, whereas this protection would have been provided by adrenergic drugs, histaminolytics, and aminophyllin. Although epinephrine did not appear to augment adrenal cortical activity, it continued to relieve asthmatic manifestations in our patients for variable but relatively brief periods. It would therefore appear that epinephrine acts directly as a bronchodilator.

Although our experience with this group of patients does not support the thesis that administration of epinephrine over a long period commonly leads to the loss of its bronchodilator effect, it is nevertheless a fact that asthmatics occasionally have exacerbations which are not relieved by epinephrine. During these exacerbations they may require more vigorous management with longer-acting preparations such as hypnotics or demerol. This does not necessarily indicate a state of tolerance in which the bronchial musculature is unresponsive to epinephrine, but suggests rather that the disease itself has become more severe, thereby increasing or altering the therapeutic requirements. When the cycle of severe asthma is broken by these measures, the condition again becomes responsive to less heroic therapy.

As has been observed by others,[4] the correlation between the clinical response to ACTH therapy and the laboratory evidence of augmented adrenal cortical activity is not necessarily close. Two of our patients were not benefited. Both showed depression of sweat sodium levels; one showed the anticipated eosinopenia, while the other did not. So here there were demonstrable adrenal cortical effects in the absence of clinical response, the converse of the frequently observed clinical response in the absence of pronounced adrenal cortical effects.

SUMMARY AND CONCLUSIONS

The effects of prolonged administration of epinephrine on adrenal cortical activity in the human being were studied in five chronic asthmatic patients.

The hypothalamus-pituitary-adrenal cortex mechanism did not appear to be in a state of chronic stimulation. Adrenal cortical function, as indicated by the effect of ACTH on the level of circulating eosinophils and the concentration of sweat sodium, was, in general, unimpaired. Thus, the ability of the adrenal cortex to respond was not apparently altered by the repeated administration of epinephrine, despite the fact that the mechanism was refractory to epinephrine.

Temporary discontinuation of epinephrine did not lead to an appreciably enhanced antiasthmatic effect when the drug was restored.

Under the stated conditions of epinephrine administration there was no evidence of the development of tolerance.

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INTRARENAL VASCULAR RESPONSES TO SYMPATHETIC STIMULATION AND TO ADRENALIN

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INTRODUCTION

ALTHOUGH it is now possible to measure accurately renal circulation[1, 2] and even to estimate certain aspects of intrarenal hemodynamics,[3-7] the data rendered by those methods show only circulatory changes in the kidney as a whole. No information is gained through them regarding circulatory variations in the different functional portions of the kidney, nor in the corresponding vascular segments.

The report of Trueta and associates[8] has shown the need for detailed knowledge regarding the process of neurohumoral regulation of the circulation in the different vascular segments of the kidney. In this paper we are reporting some of the responses of the intrarenal blood vessels to sympathetic stimulation and to Adrenalin, as revealed by injections of India ink. Blood vessels visualized by this simple method provide a picture of what is going on in the animal at a given moment.

METHOD

The observations were carried out in thirty-five cats weighing 1.5 to 3 kg., anesthetized by peritoneal injection of 0.7 ml. of Dial per kilogram of body weight. Through a medial laparotomy one of the kidneys was exposed, usually the left. The renal nerves entering the kidney with its artery were faradized unilaterally with different intensities of current, by means of a Harvard inductorium with a 1.8 volt dry battery in its primary. The nerves of the left kidney were stimulated, the opposite kidney being used as a control. Adrenalin was injected intravenously in doses varying from 2.0 to 11.6 gamma per kilogram of body weight. Electric stimulation was started twenty to thirty-five seconds previous to the injection of India ink and maintained until both kidneys had been removed. When Adrenalin was used, it was injected thirty seconds previous to the injection of the pigment. India ink was always given by arterial injection and usually forcibly injected against the blood flow into the left common carotid artery; sometimes the ink was likewise injected into the superior mesenteric artery, and in a few instances it was injected directly into the abdominal aorta, above the origin of the renal arteries. At the end of the injection, both kidneys were rapidly removed by transectioning the renal pedicle without ligating or clamping it. The removal of the kidneys was usually completed less than thirty seconds after the injection of the India ink.

Transverse sections of the kidney were immediately placed in 10 per cent formalin and fixed for a minimum of ten days. Frozen sections 60 to 100 microns thick were made and dehydrated in alcohol and xylene. They were then transferred to creosote, mounted

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on large glass slides, and covered with balsam. In order to avoid excessive shrinkage of the sections, dehydration was started in 40 per cent alcohol and carried through progressive concentrations to absolute alcohol, the sections remaining in each solution for twelve hours.

RESULTS

Controls.—Two animals which were not stimulated were injected with India ink in the manner outlined. Both cortex and medulla were stained, although the cortex was stained more heavily (Fig. 1). The interlobular arteries were entirely filled. All afferent arterioles and glomeruli throughout all areas of the cortex contained India ink. Empty glomeruli were not seen, although in many glomeruli a few capillary loops contained no ink. The intertubular capillary network was clearly outlined throughout the cortex. We consider this network as an index of the degree of contraction or relaxation of the efferent arterioles, which is very difficult to estimate directly. Some of the vasa rectae in the medulla were empty; the remainder were only partially filled (Fig. 2). A similar picture was usually seen in the control kidney in the experimental series.

Experimental.—

(a) *Sympathetic stimulation:* Low intensities of stimulation (secondary at 10 cm. and sometimes at 9 cm. from the primary) did not produce any changes in kidney circulation detectable by gross or microscopic examination.

Medium intensities (secondary at 9 to 6 cm. from the primary) produced subtotal ischemia shown by incomplete filling of the blood vessels of the stimulated kidney. Grossly, the picture was that of uniform but somewhat less intense staining than the controls. However, the microscopic pattern was variable. In the majority of instances, although the interlobular arteries appeared filled with ink in the greater portion of their length (Fig. 3), not all the afferent arterioles originating from these interlobular arteries were filled. There was a random distribution of full and empty afferent arterioles throughout the cortex (Fig. 4). The same irregular distribution of stained and empty glomeruli was observed, without any particular arrangement. Both full and empty glomeruli could be seen in the corticomedullary junction as well as in the superficial layers of the cortex. Although variations in individual responses were obvious, in general the number of ischemic glomeruli was directly related to the intensity of stimulation: greater intensities produced a more marked vasoconstriction (Fig. 5), while lesser intensities were attended by a smaller number of ischemic glomeruli (Fig. 6). The filling of glomeruli appeared to be independent of the response of the corresponding afferent arteriole. Thus, empty glomeruli were found arising from either empty or patent afferent arterioles. Fig. 7 shows two glomeruli, one stained and the other empty, arising from the same arteriole.

The intraglomerular distribution of ink was also found to be variable. Completely filled or empty glomeruli were found adjacent to others only partially filled with ink (Fig. 8). The number of these partially filled glomeruli varied considerably in different animals. The number of patent

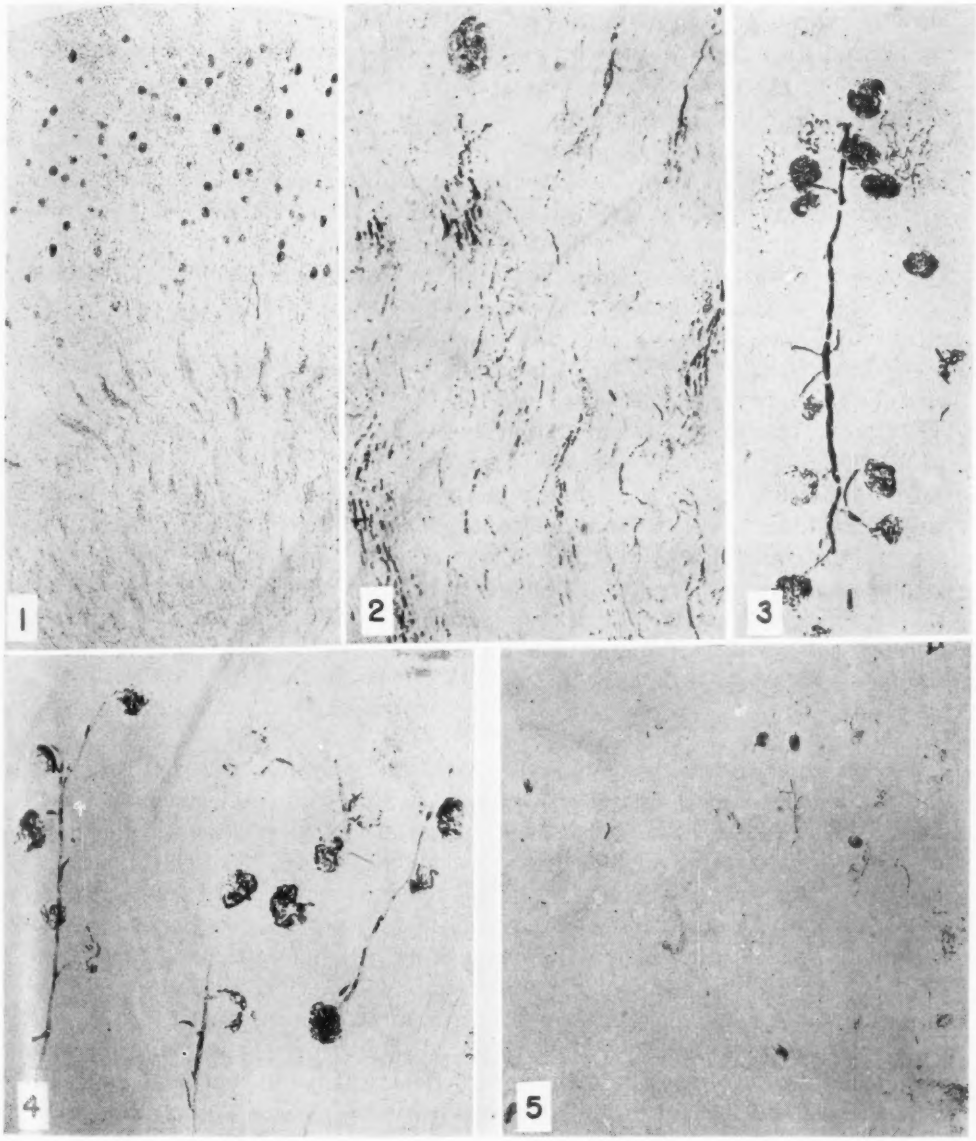

Plate I.—All figures are photomicrographs of sections of kidneys of cats injected intravenously with India ink.

Fig. 1.—Kidney of control animal. (×100.)

Fig. 2.—Kidney of control animal. Not all the vasa rectae in the medulla contain India ink. (×200.)

Fig. 3.—Kidney of cat stimulated with medium intensities of electric current. Filling of interlobular arteries but not all afferent arterioles and glomeruli contain ink. (×190.)

Fig. 4.—Same kidney as in Fig. 3, showing that closure of afferent arterioles occurs at any height in the cortex. (×190.)

Fig. 5.—Kidney of a cat stimulated with stronger intensities than the one in Figs. 3 and 4. Notice a less number of injected glomeruli. (×100.)

capillary loops as well as their relative position in a given glomerulus was also very variable.

The distribution of the cortical intertubular capillaries was irregular. However, in the cortex, there were areas in which glomeruli were partially or completely filled which were surrounded by an intertubular capillary network (Fig. 9). Within these areas the glomerular capillaries and intertubular capillaries were filled to the same degree. In addition, isolated glomeruli were seen in which the capillaries were more or less completely filled; these glomeruli were not surrounded by a filled intertubular network.

In six animals the ischemia was partial; this result was obtained after either type of stimulation. There were stained cortical areas of varying size alternating with others without any ink. Microscopically, there was a complete filling of all the vascular elements in the dark area, in contrast with the absence of ink in the empty zones (Fig. 10).

Higher intensities of stimulation (secondary at 3.5 to 5 cm. from the primary) produced complete ischemia.

(b) *Adrenalin:* In two animals receiving 2 gamma per kilogram of body weight the kidneys showed a moderate increase in the amount of ink over that seen in the controls; the distribution was normal. Higher doses (up to 11.6 gamma per kilogram) resulted in subtotal ischemia of variable degree such as that obtained after electrical stimulation of the sympathetic nerves with currents of medium intensity. Microscopic examination revealed vascular responses similar to those already described.

DISCUSSION

Glomerular intermittence described in the frog by Richards and Schmidt[9] in 1924 and by Bieter[10] in 1930, has not been proved in the mammals. Eckehorn[11] described the same phenomenon during in vivo observation of glomeruli of *Rana esculenta* and calculated that about one-tenth of the total number of capillaries was opened to the circulation at a given time. The fact that all glomeruli in our control animals were stained supports the opinion that, in the mammal, the blood circulates simultaneously through all such structures. White[12] (1939) has made similar observations. Therefore, as Smith[13] (1943) contends, there is no glomerular reserve in the sense of inactive glomeruli.

Some recent observations[14-16] seem to indicate that it is possible to increase the glomerular filtration rate with a proportional change in TmG and TmPAH. Our observations of empty capillary loops, very conspicuous in some glomeruli, would suggest that blood is not circulating simultaneously through all the capillary loops under the present experimental conditions. Therefore, although there is no evidence in the mammal for glomerular intermittence in the sense of completely inactive glomeruli, there are indications of intermittence at an intraglomerular level.

Intermittence at a subglomerular level may well be similar to the phenomenon designated by Chambers[17] as "vasomotion." Rhythmic contraction of the precapillary segment of arterioles has been observed in other areas studied in vivo, i.e.: the subungueal and mesoappendix capillaries,[18, 19] the hepatic

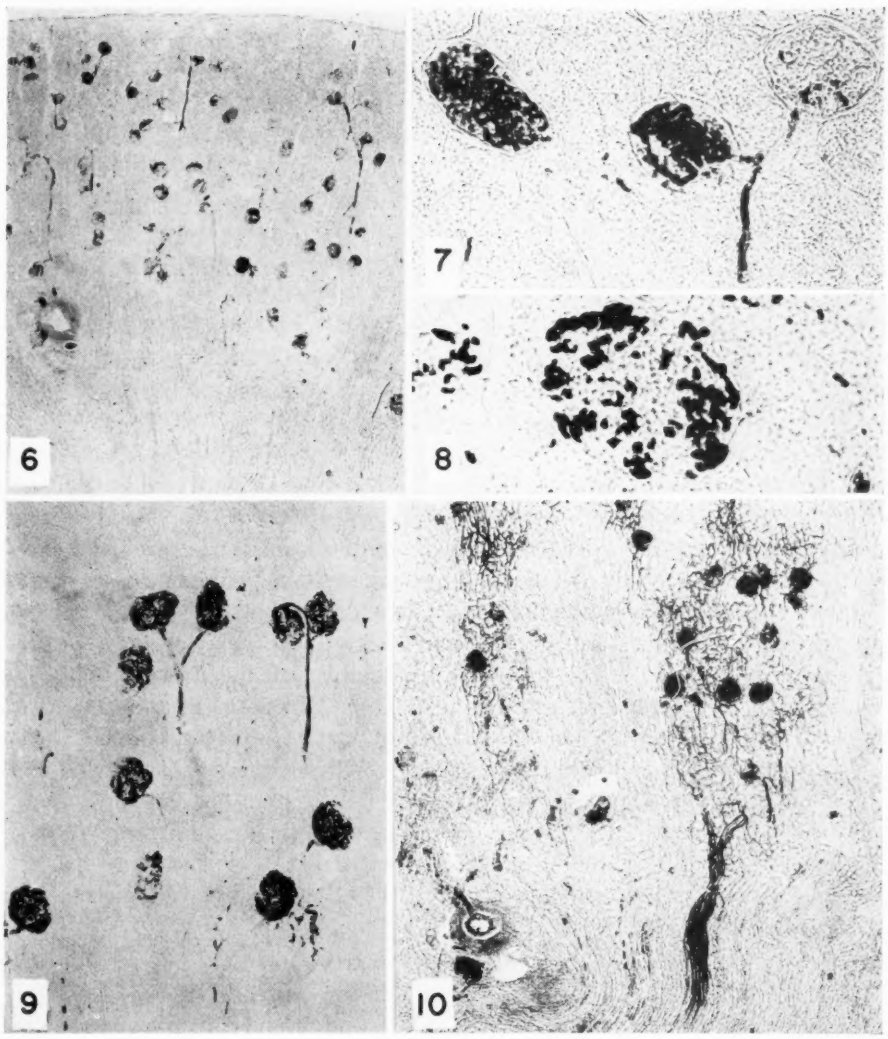

Plate II.—

Fig. 6.—Kidney of a cat stimulated with lesser intensities than the one in Fig. 5. The number of injected glomeruli is larger. (×100.)

Fig. 7.—One injected and one empty glomerulus arising from the same afferent arteriole. (×260.)

Fig. 8.—Partial filling of a glomerulus. Notice that the intertubular capillary network also contains ink. (×300.)

Fig. 9.—Glomeruli filled with India ink, some surrounded by injected intertubular capillary network and some by empty capillaries. (×190.)

Fig. 10.—Partial ischemia involving both cortex and medulla, adjacent to well-injected areas. (×180.)

sinusoids,[20, 21] the capillaries of the conjunctiva,[22] etc. Differences in the excitability of the various vascular segments of the kidney at a given moment are suggested by the fact that different intensities of stimulation evoked different degrees of vasoconstrictor response. The interlobular arteries apparently have the lowest excitability; they were found closed throughout the kidney only after very strong stimuli. With less intense stimulation, these vessels either remained open throughout their length or completely closed. Partial filling and especially partial filling of the proximal segment was never observed in these experiments. Our observations do not support the claim of Trueta and co-workers,[8] for the shunt of the blood flow through the juxtamedullary glomeruli produced by the selective constriction of the vessels of the renal cortex. This may account for the inability of numerous workers to reproduce Trueta's findings.[23-30] The few existing reports describing intrarenal vascular shunts[31-34] can be explained as a passive postischemic recovery of the circulation.[35-37] Furthermore, Breed, Maxwell, and Smith,[38] studying human beings under several of the normal and abnormal conditions mentioned by Trueta, did not find any evidence of juxtamedullary circulation.

Afferent arterioles showed a higher degree of excitability than the interlobular arteries. However, a given stimulus did not evoke the same response to all afferent arterioles. Thus, after stimuli of moderate intensity, some afferent arterioles contained ink while others in close proximity were empty. The fact that sometimes the afferent arteriole was stained while the corresponding glomerulus was empty indicates that the arteriole can contract at its glomerular end. The unequal excitability of the afferent arterioles accounts for the fact that during neural or Adrenalin stimulation, some glomeruli were excluded from the circulation while others remained patent. Indeed, increasing intensities of stimulation will occlude increasing numbers of glomeruli. Consequently the decrease of glomerular filtrate and effective renal blood flow observed after injection of Adrenalin can be explained by the complete exclusion from the circulation of a number of nephrons, as has been recently shown by Moyer and Handley,[39] rather than by a uniform decrease in activity of all glomeruli (Smith[2]).

The highest degree of excitability was observed in the efferent arterioles, as shown by the absence of India ink in the intertubular capillary network, while the corresponding afferent arterioles and glomeruli were completely stained. Stimuli unable to produce contraction of the afferent arterioles were sufficient to cause closure of the efferent vessels.

The fact that empty capillary loops were found within a given glomerulus adjacent to others well stained seems to be due to the contraction of some secondary branches of the afferent vessel, i.e., those within the glomerular tuft. These secondary branches would correspond to the meta-arterioles of other areas with their precapillary sphincters. According to Zweifach,[19] such sphincters are found in all tissues regardless of their structural organization. Indeed, the closure of some capillary loops would not stop the blood flow through the remaining glomerular capillaries into the intertubular network.

Furthermore, it is improbable that the absence of ink in the empty glomerular capillaries is due to obstruction by the ink, since the material used is a homogeneous and stable suspension of very fine particles. Therefore, it is possible that the branches of the afferent vessel within a single glomerulus have different degrees of excitability.

The partial alternate type of ischemia also observed by Houck[29] may be interpreted as the result of a limited stimulation of the corresponding nerve fibers closer to the stimulating electrode.

The greater amount of ink observed in the two cases in which small doses of Adrenalin were used may be due to the vasodilator effect of the drug in low concentration[40, 41] or to the initiation of efferent arteriolar constriction with a consequent increase in intraglomerular pressure; the latter supposition would accord with our concept of the high degree of excitability of the efferent arteriole. The larger doses of Adrenalin used in these experiments did not produce total ischemia, but much larger doses or potentiation of smaller doses of Adrenalin in kidneys sensitized by postganglionic denervation[42] should result in total ischemia.

SUMMARY AND CONCLUSIONS

The effect of the electric stimulation of the renal nerves and of Adrenalin on the intrarenal blood vessels of the cat has been studied by means of arterial injection of India ink.

Although the experimental results confirm the patency of all the glomeruli throughout the kidney, there are indications of intermittent blood flow at an intraglomerular level. The number of empty capillary loops can be increased by mild sympathetic stimulation or Adrenalin.

Different intrarenal vascular segments show varying degrees of excitability. In increasing order these are the interlobular arteries, the afferent arterioles, the glomeruli, and the efferent arterioles.

No evidence has been found to support the suggestion that the interlobular arteries are more excitable in the distal than in the proximal (juxtamedullary) segments.

The variable pattern of glomerular filling found during sympathetic stimulation is partially due to differences in excitability of the afferent arterioles which can constrict simultaneously in the deeper and the superficial layers of the cortex.

Small doses of Adrenalin can produce slight dilatation of intrarenal blood vessels.

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SEROLOGICAL REACTIONS IN RHEUMATOID ARTHRITIS

IV. Persistence of the Serum Factor Which Agglutinates Sensitized Sheep Cells

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NUMEROUS reports have confirmed the observation of Waaler[1] and Rose and associates[2] that the serum of many patients with rheumatoid arthritis enhances the specific agglutination of sheep cells to a greater degree than the serum of nonarthritic individuals. Although the diagnostic value of the phenomenon is limited by the failure of its occurrence in some cases of clinically typical rheumatoid arthritis and by its occasional appearance in other conditions,[3-9] its possible relation to the etiology and pathogenesis of rheumatoid arthritis invites further investigation. The consistent finding of some patients whose serum does not agglutinate sensitized sheep cells to an unusual degree suggests either that the property fluctuates during the course of the disease or that some individuals persistently fail to exhibit the reaction. It was of interest, therefore, to determine the titers for sensitized sheep cells of repeated blood specimens from a series of patients with rheumatoid arthritis and to attempt to correlate these results with the clinical status of the patients.

MATERIALS AND METHODS

Patients' Serum.—Specimens of serum were stored at −20° C. without preservative and were inactivated at 56° C. for thirty minutes just before testing. Sheep cell agglutinins were absorbed by adding 2 ml. of a 1 in 5 dilution of serum to 0.5 ml. of packed sheep cells, mixing thoroughly and allowing the mixture to remain overnight in the refrigerator.

Sheep Erythrocytes.—Blood from one sheep was used for all the tests in this series. Blood was stored in an equal volume of Alsever's solution for periods up to four weeks. Cells were washed three times with saline before being used for absorption or agglutination.

Hemolysin.—Antisheep cell serum was prepared by giving a rabbit five weekly intravenous injections of 0.5 ml. of packed cells suspended in saline. A stock 1 in 50 dilution of serum was prepared in saline and stored at −20° C. Further dilutions were made from this stock as required. The agglutinin titer of the serum, recorded in terms of final dilution, was determined by adding 0.5 per cent sheep cells to dilutions of serum increasing in increments of 1,000 in 0.5 ml. of saline. A fresh pipette was used for each dilution. The average titer of the hemolysin used throughout these experiments was 1 in 2,500.

The Test.—Serial doubled dilutions of inactivated, absorbed patient's serum from 1 in 10 to 1 in 2,500 were prepared in 0.5 ml. of saline. To each tube was added 0.5 ml. of 0.5 per cent sensitized sheep cells. A control consisting of patient's serum 1 in 10 plus unsensitized sheep cells was included with each serum tested. A second control consisting of sensitized cells and saline was provided for each group of tests.

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Sensitized cells were prepared by combining 10 per cent sheep cells and diluted hemolysin in such proportions as to give in the final test 0.25 per cent sheep cells and a 1 in 10,000 dilution of hemolysin. This sensitizing dilution of hemolysin was such that a standard pool of rheumatoid arthritis sera gave a titer of 1 in 640 for cells sensitized in this manner. A titration of this standard serum was included with each series of tests and showed the same titer on each examination.

All tests were incubated in a waterbath at 37° C. for two hours and placed in the refrigerator overnight. They were read immediately after removal from the refrigerator.

RESULTS

The study included observations on 63 patients (Table I). In 54, the clinical diagnosis was rheumatoid arthritis and in 9, rheumatoid spondylitis was the predominant clinical feature. From 2 to 6 serum specimens from each patient were examined. The interval between the first and last specimens was from four to twenty-nine months. In order to minimize the chance for technical variation in the results all specimens from one patient were tested at the same time after storage at –20° C.

TABLE I. CHANGES IN TITER OBSERVED IN 63 PATIENTS OVER PERIODS OF FOUR TO TWENTY-NINE MONTHS

CLINICAL DIAGNOSIS	PERIOD OF OBSERVATION	NUMBER OF PATIENTS	TITER UNCHANGED	TITER INCREASED	TITER DECREASED
Rheumatoid	4 - 11 mo.	20	14	3	3
Arthritis	12 - 20 mo.	21	18	1	2
	21 - 29 mo.	13	8	4	1
Subtotal		54	40	8	6
Spondylitis	4 - 11 mo.	1	1	0	0
	12 - 20 mo.	5	4	1	0
	21 - 29 mo.	3	3	0	0
Subtotal		9	8	1	0
Total		63	48	9	6

Table I shows the frequency with which changes in titer were observed among those followed for various time intervals. For the purpose of analyzing the data, the titer was considered unchanged if only a twofold increase or decrease was observed. Forty of the 54 patients with rheumatoid arthritis showed no significant change in titer during the period of observation. Changes were observed most frequently (5 of 8) in the group followed for the longest time, twenty-one to twenty-nine months. Only 1 of the 9 patients with spondylitis showed a significant change.

The magnitude of the changes observed is shown in Fig. 1. By plotting for each patient the titer obtained at the beginning of the period of observation against the subsequent titer showing the greatest change, points falling outside the area representing a one-dilution difference in titer are readily noted. Only one patient showed as much as a sixteen fold change, a decrease from 1,280 to 80. Fig. 1 also shows that 10 patients with rheumatoid arthritis never showed titers above 80 which was the maximum observed in normal individuals using the same procedure.

Of the 21 patients who originally showed titers of 80 or less, 11 had titers of 160 or more on subsequent specimens. On the other hand, of 33 patients

who were originally above the normal range, only 6 fell below 160 during the period of observation. Only 3 of the 9 patients with spondylitis showed titers above 80.

The relation of the titer for sensitized sheep cells to the severity of the disease is analyzed in Table II. Each case was rated from + to ++++ according to the activity of the disease based on criteria accepted by the American Rheumatism Association.[19] The cases in each category are distributed according to titer. The proportion of patients showing titers above the normal level of 80 increases regularly with increasing severity from 29 to 78 per cent. It appears, therefore, that the more severe the disease, the greater the chance that sensitized sheep cells will be significantly agglutinated.

TABLE II. TITERS FOR SENSITIZED SHEEP CELLS ACCORDING TO SEVERITY OF RHEUMATOID ARTHRITIS

SEVERITY OF ILLNESS	NUMBER OF PATIENTS	DISTRIBUTION ACCORDING TO TITER FOR SENSITIZED SHEEP CELLS								PER CENT ABOVE NORMAL RANGE
		40	80	160	320	640	1,280	2,560	5,120	
+	7	1	4		1		1			29
+ +	6		3	3						50
+ + +	22	3	6	8	4		1			59
+ + + +	19	1	3	3	5	2	3	1	1	78
Total	54	5	16	14	10	2	5	1	1	61

Classifying the titers obtained according to the duration of the disease (Table III) shows essentially the same proportion of significant reactions in patients whose symptoms have been evident for less than a year as in those whose illness had continued for many years. No patient who had been ill for less than three years, however, showed a titer above 320. Other observers[20] have recorded an increasing proportion of significant reactions with increasing duration of disease.

An attempt was made to correlate observed changes in titer for sensitized cells with changes in the clinical status of patients during the period of observation (Table IV). Changes in titer in both directions were observed among patients whose clinical condition remained unchanged, among those whose disease continued to progress, and among those who showed improvement. No

TABLE III. TITERS FOR SENSITIZED SHEEP CELLS ACCORDING TO DURATION OF RHEUMATOID ARTHRITIS

DURATION OF ILLNESS	NUMBER OF PATIENTS	DISTRIBUTION ACCORDING TO TITER FOR SENSITIZED SHEEP CELLS								PER CENT ABOVE NORMAL RANGE
		40	80	160	320	640	1,280	2,500	5,120	
0 - 11 mo.	8	1	2	3	2					63
1 - 2 yr.	7		3	3	1					57
3 - 5 yr.	16	1	6	4	2		2	1		56
6 - 9 yr.	9	1	2	1	2		2		1	67
10 - 30 yr.	14	2	3	3	3	2	1			64
Total	54	5	16	14	10	2	5	1	1	61

form of therapy including gold, testosterone, and supportive treatment appeared to have any effect on the ability of patients' serum to agglutinate sensitized sheep cells.

Perhaps of greatest interest are those patients with rheumatoid arthritis whose sera at no time contained increased amounts of the factor which causes agglutination of sensitized erythrocytes. The 10 patients whose sera never showed titers above 80 are listed in Table V. There is nothing consistent about

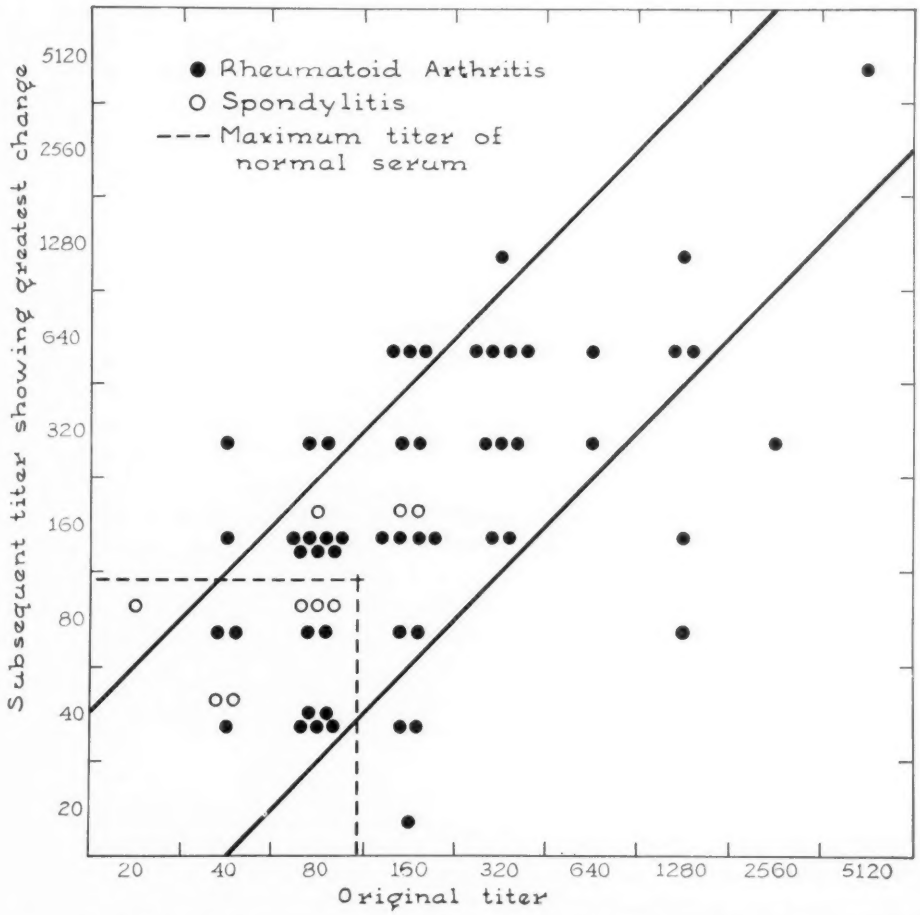

Fig. 1.—Changes in titer of patients' sera for sensitized sheep cells during periods of four to twenty-nine months.

these individuals with respect to sex, age, duration of the disease, or its severity. In 4 of these patients, in spite of severe symptoms and a condition which had persisted for at least five years, there was no increased agglutination of sensitized cells.

DISCUSSION

Since the main objective of this study was to detect possible changes in the capacity of patients' serum to enhance the agglutination of sheep cells by

TABLE IV. CHANGES IN TITER IN RELATION TO CLINICAL CONDITION

CLINICAL CONDITION	TITER UNCHANGED	TITER INCREASED	TITER DECREASED
Unchanged	14	4	1
Progressive disease	22	3	1
Improved	12	2	4
Total	48	9	6

TABLE V. RHEUMATOID ARTHRITIS PATIENTS WHOSE SERUM REPEATEDLY FAILED TO SHOW A SIGNIFICANT TITER FOR SENSITIZED SHEEP CELLS

PATIENT	SEX	AGE	DATE	TITER	DURATION OF SYMPTOMS	THERAPY	SEVERITY	CLINICAL COURSE
78	F	28	4-11-49 4-25-50	80 40	3 months	Supportive	+	Unchanged, effusion of knee
183	F	59	1-16-50 3-25-50 5- 1-51	80 80 40	1 year	Supportive	+++	Progressive
81	M	30	4-15-49 6- 9-49 8-15-49 3-17-50	80 40 80 40	2 years	Testosterone	+	Generalized arthritis, in remission following therapy
28	F	61	12-22-48 1-11-50	80 40	5 years	Gold	+++	Generalized arthritis, in remission following therapy
56	M	14	2-14-49 11-14-49 3-23-50	80 40	5 years	Supportive	+	Unchanged, effusion of knee
64	F	53	3- 7-49 8-15-51	40 40	5 years	Supportive	+	Unchanged
150	M	60	9- 8-49 4-27-50	80 80	5 years	Supportive	++++	Progressive
36	F	50	12-30-48 1- 9-49 9- 7-49	80 80 80	10 years	Gold	++++	Progressive
113	F	73	6- 9-49 1-11-50	40 80	10 years	Supportive	++++	Progressive
131	F	60	8- 3-49 1-13-50	40 80	20 years	Supportive	+++	Unchanged

specific antibody, it was essential to eliminate from the procedure as many variables as possible. An attempt to use the data accumulated over a period of two and one-half years proved to be unsatisfactory. During this time different antisheep cell sera were used, sheep cells were obtained from several different animals, and several individuals had been concerned with the performance and reading of the tests. All these variables were eliminated by testing the entire series of specimens from one patient at one time. Previous observations have failed to show loss of activity on storage.[11, 12] The accuracy of the results was further controlled by including a pooled rheumatoid arthritis serum with each batch of tests. The same titer was obtained with this serum on each of the 8 times the tests were performed. That several factors may affect the reproducibility of the results has been pointed out by Pike, Sulkin, and Coggeshall,[11] Winblad, [13] Scott,[9] and Svartz and Schlossmann.[8]

Few observations have thus far been reported concerning the occurrence of the agglutination enhancing factor in patients with rheumatoid arthritis over a period of time. Svartz and Schlossmann[8] observed that the test used by them did not become positive for four to eight weeks after the onset of symptoms, and that the titer did not necessarily change with clinical improvement. Wager[7] found a significant reaction in 65 per cent of patients showing symptoms for less than two years, and Winblad[14] reported positive reactions in 42 per cent of patients with symptoms for one year or less. On the other hand both positive and negative reactions have been observed in patients with symptoms of rheumatoid arthritis for ten to twenty years.[7] Of 5 patients followed by Waaler[1] for one year, 2 showed strong reactions with no change, 2 showed strong reactions which decreased, and 1 with a slight reaction became negative.

The proportion of patients with rheumatoid arthritis showing increased agglutination of sensitized sheep cells varies greatly in different reports. Svartz and Schlossmann[8] obtained significant reactions in about 90 per cent of their patients. Heller and others,[15] taking advantage of the potentiating effect of sheep serum on the reaction, increased their proportion of positive tests from 75 per cent to 85 per cent. Others have recorded significant reactions in as few as 23 per cent.[3] Invariably, however, some patients in every series show reactions within the normal range. It is yet to be learned whether or not these are individuals with rheumatoid arthritis who never develop the capacity for increased agglutination of sensitized cells. Our results show that on repeated examinations a higher proportion of positive results are obtained. Of the patients studied here 39 per cent were within the normal range on the first examination. Only 19 per cent, however, consistently showed titers of 80 or below on repeated examination.

Our failure to note any correlation between therapy and change in titer in these patients is consistent with the observation that the agglutination of sensitized sheep cells is not altered by treatment with ACTH and cortisone.[8, 16, 17, 18, 19]

SUMMARY

Repeated serum specimens from 54 patients with rheumatoid arthritis and 9 patients with rheumatoid spondylitis were examined over periods up to twenty-nine months in order to detect possible changes in the capacity of the serum to agglutinate sensitized sheep cells. Forty of the patients with rheumatoid arthritis and 8 of those with spondylitis showed no significant change. There was no apparent relation between therapy or change in clinical symptoms and change in the titer of the serum. The titers in 6 patients with rheumatoid arthritis remained within normal limits in spite of severe or progressive disease.

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STUDIES ON ANTIBODIES ELUTED FROM THE RED CELLS IN AUTOIMMUNE HEMOLYTIC ANEMIA

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THE diagnosis of acquired hemolytic anemia of the autoimmune variety now rests firmly on the laboratory demonstration of an abnormal immunologic mechanism. The detection of antibodies has been greatly facilitated by techniques derived from work in the field of the hemolytic disease of the newborn infant. These include the use of trypsinized erythrocytes as test cells[16, 19] and the use of protein rich media as diluents.[6, 17] Because of the peculiarities of autoimmunization, both the deleterious agent (the antibody) and the target organ (the erythrocyte) exist within the same individual. On theoretical grounds, it might be anticipated that most commonly a complex consisting of antigen and antibody would be found, since the large excess of antigen (i.e., the erythrocytes), present would join with the antibody as the latter was produced. In many cases, this appears to be so as no free antibody can be detected in the serum. This is in contrast to the uniformly successful detection of antibody adherent to the red cell surface. Demonstration of this adherent antibody may be made by the use of anti-human globulin serum[1, 3, 4] (Coombs' test) or by elution of the antibody from the red cell surface.[5, 9, 13, 20, 22]

The mere elution of antibody from the red cell surface would in itself be of no value were it not possible to demonstrate the action of the antibody contained in the eluates. Since the antibody in autoimmune hemolytic anemia is usually of the "incomplete" variety, elution into saline solutions, as generally practiced, will not demonstrate activity without further manipulation. By application of the Coombs' test or the use of trypsin reaction, it has been possible to detect even small amounts of antibody activity in such saline eluates.

Although studies of eluted antibodies in autoimmune hemolytic anemia have been made previously by several observers, the present study carries this work further along with particular reference to the following three points:

1. A comparison of the results obtained with elution and by the Coombs' technique in the demonstration of antibody attached to the red cell.

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2. A comparison of the trypsin method with that of the indirect Coombs' technique for revealing the presence of antibody in the eluates.
3. A study of the range of activity (specificity) of the antibody obtained in the eluates.

METHODS AND MATERIALS

Case Selection.—Sixteen cases of acquired autoimmune hemolytic anemia were studied, four being secondary to chronic lymphocytic leukemia and twelve being "idiopathic" in type. All cases showed at one time or another the classical findings of hemolytic anemia, namely, pallor, icterus, and splenomegaly together with such laboratory evidences as anemia, reticulocytosis, and indirect hyperbilirubinemia. In all cases the direct Coombs' test was positive. Fifteen of these cases were examined by the albumin and trypsin techniques for the presence of free red cell antibodies in the serum. Only four of them gave negative results. The free antibodies in the remaining eleven cases were present in low titer.

Collection of Specimens.—Venous blood was drawn from an antecubital vein with a minimum of stasis by means of a dry syringe and a 20-gauge needle. The blood was immediately transferred to a clean dry Ehrlenmeyer flask and gently rotated in horizontal direction with a glass rod containing a roughened tip. After ten to fifteen minutes' rotation the rod was withdrawn with its adherent fibrin clot. Filtration of the red cells in serum through loose cotton wool was usually necessary for elimination of small clots or fibrin particles.

In a few instances, citrated or oxalated blood was used. As a rule, elutions were carried out on the same day that the red cells were obtained, but on occasions this was deferred for twenty-four hours, the blood being kept in the refrigerator at 3° C. Before use, such refrigerated specimens were allowed to equilibrate at room temperature so as to remove from the red cells any possible absorbed cold agglutinins.

Preparation of Stroma.—Ten to fifty ml. of packed red blood cells derived in the above manner were washed four to six times with copious amounts of physiologic (0.85 per cent) saline. The red cells were then firmly packed by centrifugation and hemolyzed with ten times their volume of distilled water. By the dropwise addition of 1N HCl until the pH was in the range of 5.6 to 5.8, precipitation of the stroma was readily carried out. An alternative method was the use of distilled water which had been saturated with CO_2 in the cold.

The precipitated stroma was then washed repeatedly with distilled water buffered at pH of 5.6 to 5.8. It was found that the elimination of hemoglobin was most complete when the stroma was frozen and thawed in between every three or four washings. The residual stroma was then almost white or just slightly brown tinged and after firm packing was ready for elution by one of the three following methods.

METHODS OF ELUTION

1. *Elution Method of Kidd.*[13]—The stroma was suspended in a citrate-phosphate buffer at pH 3.0 to 3.2 and thoroughly mixed. An amount of buffer equal to twice the volume of packed stroma was used. After standing at room temperature for fifteen to twenty minutes, the mixture was centrifuged, the sediment discarded, and the supernatant immediately adjusted to pH 7.0 by addition of 5N sodium hydroxide. At this point a precipitate formed which was readily removed by centrifugation.

2. *Elution Method of Landsteiner.*[9, 14, 15, 20]—The well-packed stroma was suspended in twice its volume of normal saline and, after thorough mixing, was incubated at 56° C. for fifteen minutes. It was then centrifuged at high speed in prewarmed cups and the supernatant collected.

3. *Elution Method by Hypertonic Saline.*[2, 8, 10, 12]—(As suggested by Robert S. Evans.) The packed stroma was suspended in twice its volume of 8 per cent sodium chloride solution. After incubation at room temperature for one to two hours, the suspension was centrifuged and the supernatant collected. Isotonicity was then restored by dialysis against large quantities

of normal saline in the cold for forty-eight hours. Final centrifugation was always necessary for elimination of some residual turbidity.

All eluates were stored at −30° C. in well-stoppered tubes until use. Of the various methods used, somewhat more potent eluates were obtained by use of Method 1.

Testing of Eluates for Activity.—Eluate activity was determined by five separate methods.

1. *Saline Agglutinin Method:* Two-tenths milliliter of the eluate was mixed with two drops of untreated, washed red cells suspended in normal saline in a concentration of 2 per cent. The erythrocytes were either Group O, Rh negative, or Group O, Rh positive. The resultant mixture was incubated at 37° C. for two to three hours, following which examination for agglutination was made macroscopically. Fine agglutinates were graded as one plus, medium-sized as two plus; large clumps as three plus, and a solid agglutinate as four plus.
2. *Albumin Agglutination Method:*[6, 17] This was carried out in a fashion similar to the saline agglutinin method with the exception that the erythrocytes were suspended in 20 per cent bovine albumin in saline.
3. *Indirect Coombs' Method:*[3, 4] If the cells were not agglutinated by No. 1, they were then washed three to four times with normal saline and then made up to a 2 per cent suspension in normal saline. Two drops of this suspension and two drops of a potent anti-human globulin serum were mixed in a small serological tube, incubated at 37° C. for thirty minutes, centifuged at 1,200 r.p.m. for one minute, and then read for agglutination. Grading of agglutinates were similar to No. 1.
4. *Trypsinized Erythrocyte Method:*[16] This was similar to procedure 1, with the exception that a 2 per cent suspension of trypsinized cells was used in place of untreated cells.
5. *Trypsinized Erythrocyte-Indirect Coombs' Method:*[21] This procedure was carried out on all trials in which no agglutination was noted by Method 4. The trypsinized cells were well washed four times with normal saline following their period of incubation. They were then reconstituted to a 2 per cent suspension in normal saline and two drops of this suspension was mixed with two drops of anti-human globulin serum. The mixture was then incubated at 37° C. for thirty minutes, centrifuged at 1,200 r.p.m. for one minute, and then read for agglutination.

Trypsinization of Red Blood Cells.[18]—Five tenths milliliter of washed erythrocytes were mixed with 2.25 milliliter of isotonic phosphate buffer at pH 7.7 to which 0.25 milliliter of 1 per cent crystalline trypsin (Armour) in N/20 HCl was added. The mixture was incubated at 37° C. for sixty minutes and the cells were then washed two times with normal saline and made up to a 2 per cent suspension in normal saline.

Preparation of Coombs' Serum.—Coombs' serum was prepared according to the method of Emerson and associates.[7] Equal parts of Group O serum and Falba* were blended together in a mortar and to this was added an approximately equal volume of a suspension of *Mycobacterium butyricum* (1 mg. per milliliter) in mineral oil. The resultant viscous material was injected subcutaneously in one milliliter doses weekly for three injections. One month later a "booster" dose of one milliliter of Group O serum was given subcutaneously. A week later the animal was bled, the serum inactivated at 56° C. for thirty minutes, and absorbed with washed pooled red cells three times. Final effective titer was 1/40,000 with well-sensitized cells.

pH Measurement.—Measurements of hydrogen ion concentrations were made with a Beckman model G pH meter. Occasionally hydrion paper was used as an indicator.

Preparation of Boiled Stroma.—Packed stroma was suspended in five times its volume of distilled water buffered to pH 5.6-5.8. It was then boiled in a beaker for fifteen minutes.

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Absorption of Eluates with Stroma.—Well-packed boiled or untreated stroma was suspended in five times its volume of eluate and incubated at 37° C. for sixty minutes with occasional mixing. The mixture was then centrifuged and the supernatant was subjected to reabsorption in a similar fashion.

Absorption with Human and Animal Red Blood Cells.—Untreated normal human or animal erythrocytes were used for the absorptions after having been washed four to six times with normal saline. Each eluate was absorbed twice by one-tenth its volume of packed red cells, the absorption being carried out for one hour at 37° C. with occasional mixing. The human red cells used for the absorptions were group O, Rh negative. With monkey cells the absorption was performed three times and the volume of the packed cells used each time was one-fifth the volume of the eluate. Red cells from the species *Macacus rhesus (Macacus mulatta)* were used.

Controls.—Eluate prepared in the same manner from the red blood cells of normal individuals was used in each test as a control.

RESULTS

Comparison of Elution Technique with the Direct Coombs' Technique.—In sixteen cases, performance of the direct Coombs' test on the patient's erythrocytes gave positive results. It should be mentioned that in one case, at times, the direct Coombs' test was negative, although an active eluate could be obtained from the cells at this particular time. Demonstration of coating antibody by means of elution techniques was made in thirteen of the sixteen Coombs' positive cases. In the remaining three cases, although the Coombs' test indicated that absorbed antibody was present, elution failed to demonstrate it (Table I).

TABLE I. DEMONSTRATION OF COATING ANTIBODY BY DIRECT COOMBS' TEST AND BY ELUTION TEST (BY CASE)

	DIRECT COOMBS' TEST (NUMBER OF CASES)	ELUATE ACTIVITY (NUMBER OF CASES)
Positive	16	13
Negative	0	3*

Note: In one instance where Coombs' test was occasionally negative, eluate activity was quite strong. (Titer 1/64.)

*Two of these were idiopathic and one secondary to chronic lymphocytic leukemia.

Optimal Method for Demonstration of Antibody Activity in Eluates.—For the analysis of the various methods used to test antibody activity, the three cases which showed no activity by any method were excluded, leaving, therefore, thirteen cases for study. By utilizing the simple admixture of untreated cells in saline with the eluates, antibody activity could be demonstrated in only one of thirteen cases. When the cells were suspended in 20 per cent bovine albumin, antibody could be demonstrated in six of the thirteen cases. Incubation of untreated cells with the eluates followed by a Coombs' test on the cells was capable of demonstrating antibody in thirteen of the thirteen cases. Incubation of the eluates with trypsin-treated erythrocytes resulted in antibody being detected in eleven cases out of twelve in which this test was performed. Testing of the one failure by this method with a Coombs' test on the previously incubated trypsin-treated erythrocytes resulted in a positive result (Table II).

TABLE II. OPTIMAL METHOD FOR DEMONSTRATION OF ELUATE ACTIVITY (BY CASE)†

	IN SALINE	IN ALBUMIN	INDIRECT COOMBS' TEST	TRYPSIN TEST	INDIRECT COOMBS' ON TRYPSIN-TREATED CELLS
No. of cases tested	13	13	13	12*	1
Positive	1	6	13	11	1
Negative	12	7	0	1	0

*One case was not tested.
†Three cases excluded in which no method showed activity.

Analysis of the above results by the number of individual trials rather than by case indicated that the use of untreated cells in saline was successful in only one of twenty-nine trials. Use of such cells in 20 per cent albumin was successful in ten of twenty-six attempts. By use of the indirect Coombs' technique twenty-five positive results were obtained in twenty-six efforts. Slightly less efficient was the employment of trypsin-treated cells in saline, being positive in thirty-one of thirty-six trials. Application of an indirect Coombs' test on the five failures produced positive results in all. Thus as indicated in Table III, of the various methods used the combination of trypsinization of the red cells with the indirect Coombs' test proved to be the most sensitive in detecting antibody in the eluates.

TABLE III. OPTIMAL METHOD FOR DEMONSTRATION OF ELUATE ACTIVITY (BY TRIAL)

	IN SALINE	IN ALBUMIN	INDIRECT COOMBS' TEST	TRYPSIN TEST	INDIRECT COOMBS' ON TRYPSINIZED CELLS
No. of trials	29	26	26	36	5
Positive	1	10	25 (96%)	31 (86%)	5
Negative	28	16	1	5	0

Range of Activity of Antibody Obtained in Eluates.—A series of eluates known to be active (titer 16 to 32) against human cells by some or all of the above methods was tested against various erythrocytes of animal origin. These were suspended in saline in both untreated and trypsinized state and tested against the eluates in the usual fashion. Indirect Coombs' tests were also carried out on the untreated cells of rabbits, guinea pigs, and sheep following incubation with eluates. The results are expressed in Table IV. It may be seen that these eluates, although potent for human cells, were completely without action on the cells of various other mammalians. This lack of reaction between the antibody in the eluates and animal cells was confirmed by the failure of these cells to absorb antibody from the eluates. Table V demonstrates no significant reduction of titer against human cells following such absorption with animal cells. On the other hand untreated human red cells absorbed completely the antibody from the eluates so that these could not agglutinate trypsinized human cells any more. The untreated human cells that were used for the absorptions became Coombs' positive. That shows that the antibody which reacted with untreated cells was identical with the antibody which agglutinated trypsin-treated erythrocytes.

TABLE IV. ACTIVITY OF ELUATES AGAINST ANIMAL ERYTHROCYTES

		NUMBER OF ELUATES TESTED	AGGLUTINATION
Rabbit	In saline	4	-
Rabbit	Indirect Coombs'	4	-
Rabbit	Trypsin test	3	-
Guinea pig	In saline	3	-
Guinea pig	Indirect Coombs'	3	-
Guinea pig	Trypsin test	3	-
Sheep	In saline	4	-
Sheep	Indirect Coombs'	4	-
Sheep	Trypsin test	3	-
Monkey	In saline	12	-
Monkey	Trypsin test	12	-

TABLE V. AGGLUTINATING ACTIVITY OF ELUATES, THAT WERE ABSORBED WITH ANIMAL CELLS, AGAINST TRYPSINIZED HUMAN RED CELLS

	1	2	4	8	16	32
Untreated eluate	++++	++++	+++	++	+	-
Eluate absorbed with untreated human cells	+	±	-	-	-	-
Eluate absorbed with untreated rabbit cells	++++	++++	++++	++	+	-
Eluate absorbed with untreated sheep cells	++++	+++	+++	+++	+	-
Eluate absorbed with untreated guinea pig cells	+++	++	++	+	+	-
Eluate absorbed with untreated monkey cells	++++	++++	+++	+++	+	-

Similar experiments using stroma derived from untreated human cells as absorbents removed antibody activity from the eluates. When boiled stroma was used such absorption could not be demonstrated.

DISCUSSION

Since Boorman, Dodd, and Loutit[1] first demonstrated fixed antibody on the red cells of patients suffering from acquired hemolytic anemia by means of a direct Coombs' test, ample confirmation of this observation has been offered. Most convincing evidence on the efficiency of the direct Coombs' test has been afforded by the work of Sturgeon, Evans and associates, and Kidd.[9, 13, 20] These observers, by using different methods, were able to elute antibodies off the red cell surface of Coombs' positive cells in acquired hemolytic anemia. Since then, Dacie and de Gruchy[5] and Young and his group[22] have demonstrated the value of elution techniques in the study of the antibody in acquired hemolytic anemia.

The present study in agreement with the above-mentioned studies reveals that in the majority of cases of acquired hemolytic anemia with positive Coombs' test, it is possible to elute antibody from the red cell surface. We encountered three failures out of sixteen cases. Such failures may be due to three possible causes, namely (a) loss of the antibody during the process of preparing the stroma for elution, (b) failure to dissociate the antigen-antibody complex because of unusual stability, or (c) failure to demonstrate the antibody activity

in the eluate. In one case in which the direct Coombs' reaction was ocasionally negative with potent antiglobulin sera, it was possible to elute antibody from such apparently Coombs' negative cells. The studies of Hubinont[11] suggest that a false negative Coombs' test may be due to either an excess of antibody (highly potent Coombs' serum) or an excess of antigen (large amount of antibody attached to the red cells). In our case exposure of the patient's cells to serial dilutions of the Coombs' serum gave negative results. This excludes the first possibility and we think that the negative reaction was due to an excess of antigen, namely, to an excessive amount of antibody on the red cell surface.

Antibody activity in the eluates was well demonstrated by an application of the indirect Coombs' principle. Use of trypsinized erythrocytes alone was almost as successful. Finally, with the use of a combined trypsinized erythrocyte-indirect Coombs' test as suggested by Unger,[21] the results were as good as with the indirect Coombs' test alone. It must be remembered that in some cases it was necessary to prepare more than one eluate in order to demonstrate activity This was obviously not due to the lack of antibody on the cell surface but to certain inherent technical failures in either the elution or the demonstration of antibody in the eluates. Our own experience using test cells of various human blood groups in limited trials against these eluates has been in agreement with Kidd and Sturgeon,[13, 20] namely, that the eluted antibody is a panagglutinin. Thus, no differences were found when these eluates were tested against cells of the various A, B, O, or Rh blood groups. With respect to animal cells we did not find any evidence to indicate that these eluted antibodies had corresponding receptors in the blood cells of rabbits, guinea pigs, sheep, or monkeys. This is not wholly in agreement with Kidd who through the use of rabbit anti-human globulin serum found that monkey cells were weakly sensitized. Sturgeon,[20] using Coombs' serum previously absorbed with monkey cells, did not find any such sensitization of monkey cells by antibody of eluates. We have recognized the ability of untreated Coombs' serum to agglutinate nonsensitized monkey cells and for this reason we have disregarded positive results by this method when using the cells of this animal.

The failure of these animal cells to absorb antibody from the eluates would seem to strengthen the contention that these animals lack receptors for the eluted antibody. The antibody of acquired autoimmune hemolytic anemia thus seems to be wholly reactive against some receptor in the human red cell; this appears to have no relationship to the usual blood group antigens. The failure of boiled human stroma to absorb the antibody from eluates would indicate the heat lability of this receptor.

SUMMARY

The erythrocytes of sixteen cases of acquired hemolytic anemia of the autoimmune type were utilized for elution studies. All cases displayed a positive direct Coombs' test at one time or another. Antibody could be eluted from the cells of thirteen of these cases.

Activity of antibody in the eluates was demonstrated by use of the indirect Coombs' test, the trypsin test and the combined trypsin-indirect Coombs' test.

It seems that the simple indirect Coombs' and the combined trypsin-indirect Coombs' methods are a little more sensitive than the simple trypsin test.

Antibody in such eluates did not react against nor could be absorbed by cells of rabbits, guinea pigs, sheep, or monkeys.

Boiling destroyed the receptor, for these eluted antibodies, in human cells.

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PRESENCE OF ANTI-D ANTIBODY IN THE SERUM OF A D^u PATIENT

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THE occurrence of "intermediate" Rh types whose outstanding characteristic is their inability to give strong saline agglutination reactions with potent anti-D serums was first reported by Wiener.[1] Family studies revealed the hereditary nature of these groups. More detailed investigation by a number of workers[2-6] has confirmed the original observations and extended the knowledge concerning the weakly reacting, or D^u types. In reality, D^u does not refer to a single entity, for the range of these bloods extends from ones giving feeble reactions with saline test tube anti-D serums to those whose antigen can be shown only by the Coombs technique with strong blocking anti-D serums. Various gradations lie between the two extremes.

Despite their reduced antigenicity in vitro, D^u cells are fully capable of stimulating the production of antibodies. This was first shown by van Loghem.[7] Since then, cases of erythroblastosis fetalis caused by this antigen have been reported[8-10] and an instance of transfusion reactions has been traced to the D^u factor in apparently D-negative erythrocytes of the donor.[11] Strangely, in all cases the antibody produced by stimulation with D^u cells is indistinguishable from the ordinary variety of anti-D.

The known antigenicity of D^u, the close relationship of D^u to D as evidenced by the production of an identical antibody by either antigen, and the failure to find anti-D in a number of D^u obstetrical patients have led to the development of the concept that a D^u person should be incapable of producing anti-D antibody. Stratton and Renton[2] stated this indirectly when they said that D^u should be considered Rh-positive for antenatal purposes, and thought it "very improbable" that persons of even the lowest grades of D^u could make anti-D. In a personal communication, Levine expressed his opinion that this view was correct, and Rosenfield[9] relied on the premise as one means for checking the D specificity of Rh antiserums. We, too, partially accepted the logic of such a view, but not to the extent of committing ourselves to making a definitive report of Rh positive on prepartum D^u specimens. A second sample was requested in all cases for verification. If the results were reproducible, a covering letter was sent with the report explaining our opinion of the status of the D^u factor. This policy proved itself valuable when we encountered a D^u patient with anti-D in her serum.

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CLINICAL HISTORY

The patient, Mrs. J. P., was a para iii, gravida iv. Her first child was delivered in 1944 after a normal pregnancy. In 1946, a second pregnancy terminated in a miscarriage at six weeks. Two pints of Rh-negative blood were necessary at this time. A year later the woman was delivered of another normal infant after an uneventful prepartum course. Her last pregnancy began in April, 1952. At two months the patient threatened abortion, but this was averted by stilbestrol and paregoric therapy plus bed rest. Late in October contractions began, and on October 27 a premature infant of 2 pounds, 6½ ounces was born. It expired twenty-four hours later. Hospital records show that no Rh typing was done on the infant, but a direct Coombs' test on the cord blood was negative, as might be expected in view of the low titer of maternal antibody at the time of delivery. No evidence of hemolytic disease could be found. Placenta previa caused a marked loss of blood during labor, which necessitated a transfusion with one unit of Rh-negative blood. There were no sequelae.

The antibody titer was very low throughout the last pregnancy. In May, 1952, only the undiluted serum agglutinated D positive cells. Titrations at intervals showed no significant variations. Even on October 30, three days after the birth of the baby, the titer was only 1:4. No further quantitative studies were made until January, 1953. A sharp postpartum rise had occurred with a 1:256 result on a sample collected at that time. No saline agglutinins were demonstrable in any specimen. The striking increase in titer can best be explained by the presence of D in the infant. (It can be inferred that the baby was D positive in view of the presumptive homozygosity of the father as shown in Table I.)

METHODS AND RESULTS

During the fourth pregnancy, a clotted blood specimen was sent to the Blood Grouping Section of the Utah State Department of Health Laboratories for routine prenatal typing. In accordance with usual procedures, tests for the D antigen were made in duplicate. An anti-D agglutinating serum was used with a 2 per cent erythrocyte suspension by the test tube technique and an anti-D blocking serum was employed for the PVP stick test.[12] When both were negative, examinations for C, E, and D^u were made. Standard procedure for D^u involved the use of three potent commercial antiserums which had been carefully checked to establish their specificity as pure anti-D. The patient's cells in 2 per cent saline suspension were allowed to react with each serum individually, after which the Coombs' test was applied. Mrs. J. P.'s cells gave definite agglutination with two of the three serums. Even on D^u patients, testing the serum for antibody by the trypsin method[13, 14] is part of our routine. When Mrs. J. P.'s serum disclosed an antibody of apparent anti-D specificity, a second sample was requested. All of the previous findings were duplicated. A more extensive study of the antibody specificity was made, but tests against a panel of over two hundred specimens, including all of the common subgroups of Rh, permitted only the conclusion that the antibody was anti-D. Additional serums were included in the D^u study, but the diagnosis of D^u seemed inescapable. A portion of this specimen was sent to the Holy Cross Hospital Research Foundation. There the serum was tested against a panel of cde cells containing K, k, Le^a, Fy, P, and M-N antigens with completely negative results, thus confirming more definitely the results obtained with the large array of cells. They also reported D^u in the patient's erythrocytes. A separate specimen was sent to Dr. Phillip Levine who agreed that the cells contained D^u,

TABLE I. BLOOD GROUP STUDY OF J. P. FAMILY

	SYSTEM													
	A-B-O	Rh-Hr						M-N		KELL-CELLANO		OTHER		
	A-B-abs.B	C	c	D	E	e	D^u	M	N	K	k	Fy^a	Le^a	P
Mrs. J. P.	A_1	+	+	–	–	+	+	+	–	+	+	+	–	+
J. P. (husband)	O	–	+	+	+	–		+	+	–	+	–	–	+
M. P. (son)	O	–	+	+	+	+		+	–	–	+	–	+	+
G. P. (son)	O	–	+	+	+	+		+	+	–	+	–	–	+

and the serum anti-D. (Dr. Levine informs us that Dr. Richard Rosenfield reached the same conclusion with the elution technique.)

Family studies and more complete typing on the patient were done. The findings are listed in Table I.

DISCUSSION

The principle of *horror autotoxicus* appears to have been violated in this case, since the patient formed an antibody against an antigen closely related to one which she possesses in her circulation. Any exception to such a basic tenet merits some attention, although precedence exists for a situation of this kind. Wiener[15] has compiled data which show that anti-A_2 occurs in about 0.4 per cent of A_1 serums, while A_1 antibody is found in approximately 20 per cent of A_2 individuals and 25 per cent of A_2B. The A_3 subgroup is characterized by the presence of A_1 antibody in the serum.[16] CC persons can be sensitized to form C^w antibody. Both C and C^w antibody can be produced in cc individuals immunized to C antigen alone.[17] The case cited in this paper is the first of its kind in relationship to the very important D antigen, but it is by no means unique in the general field of human blood group antigen-antibody associations. In one respect the system does differ from the others mentioned in that both the major antigen (D) and the minor antigen (D^u) stimulate the production of an apparently identical immune antibody.

It could be postulated that an antibody-suppression factor might be operative, with this factor working in converse relationship to a particular antigen. Assuming that D^u is a mutation from D, the further it becomes removed from the parent stock, the more likelihood that the suppression factor would become less effective. This supposition indicates that it should be much more difficult to sensitize a high grade D^u to D than a low grade.

Another possibility is that the typical D antigen may be capable of losing some component parts without a complete destruction of specificity. For example, if the complete D antigen is made up of three parts, D1, D2, and D3, a high grade D^u might be represented as an individual who had lost one constituent, a low grade, one who lacks two. Want of the complete antigen could account for the failure of these cells to give strong reactions under ordinary methods of typing. The fact that one part of the antigen remained would explain the fact that D^u sensitization always seems to result in an antibody not distinguishable from ordinary anti-D. Following the same line of reasoning, a person possessing only D1 could become sensitized to D2, D3, or both. Since the D^u characteristic has been shown to be inheritable, some type of partial gene deletion would have to be considered to support this hypothesis. Complete gene disappearance is already known, so this may not be too implausible.

On the practical side is the question of how to classify D^u patients for the benefit of the clinician. The situation is much the same as that existing eight to ten years ago with regard to antigens C and E. When first introduced to this field, we remember being told that the ideal Rh antiserum would contain antibodies against C, D, and E, since in that way, all Rh positives could be eliminated from consideration as possible antibody producers. Subsequent developments showed that C and E individuals were just as susceptible to D as were cde. Since then, persons of type C or E have had a dual role: as

donors, they are Rh positive; as obstetrical patients, Rh negative. As it appears now, any D^u donor and any D^u husbands whose wives are D negative should be considered Rh positive. On the other hand, D^u pregnant women should be classified Rh negative. The analogy between the C and E groups and D^u ends at this point. The former are easy to identify but D^u offers much more difficulty.

The question arises concerning qualitative differences between related antigens such as D and those of the D^u series. How much variation between any two antigens is necessary before one can stimulate the production of antibody in a person possessing the other? It is conceivable that we may some day be able to establish a series of spectral bands for D^u and find that cross-sensitization between different portions is possible. No such pattern is evident yet.

The case cited in this paper concerns a low grade D^u and it seems logical to assume that a person whose antigen was quite far removed from the D variety should be most easily sensitized to the powerful D antigen. Actually, this is merely a guess, and until more data are available concerning these subgroups, we have adopted the policy of considering any blood showing a 1+ or less reaction with good saline test tube serums as Rh negative for prenatal purposes, regardless of its reaction with other techniques and serums. Needless to say, any grade D^u is Rh positive if a husband or donor is involved.

SUMMARY

A case is presented in which a D^u obstetrical patient developed anti-D antibody in her serum. Theoretical considerations and practical aspects are discussed.

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THE EFFECT OF HEPARIN ON THE FORMATION OF P^{32}-LABELED PHOSPHOLIPID

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THE accumulated clinical and experimental evidence has indicated that atherosclerosis is associated with, or caused by, an error in lipid metabolism. Recently it has been shown that heparin can produce profound alterations in the lipoprotein spectrum of human sera. Following the use of this agent, marked reduction in the symptoms of angina pectoris[1a] has been reported, and others have observed that heparin is capable of clearing the alimentary lipemia in human beings.[2] Experimental animals subjected to high cholesterol diets are protected from atherosclerosis by the administration of heparin.[1] This study was conducted to learn whether heparin would affect the rate of radioactive phospholipid synthesis in the human being and thus be shown to affect lipid metabolism directly.

Lipid metabolism in patients with atherosclerosis has been shown to be abnormal by several methods of study. However, it has not been possible to demonstrate that these derangements are directly related to the development of human atherosclerosis. Actually the vascular alterations and lipid abnormalities may both be the result of an underlying disturbance in cellular function, as yet unrevealed. If this is correct, the various deviations in lipid metabolism from the normal may represent, in some instances, an index of the vascular disease or the predisposition to it. Among the abnormalities described in serum lipid in patients with atherosclerotic disease are: (1) hypercholesterolemia, (2) a lowering of the phospholipid cholesterol ratio, (3) a high concentration of the S_f 12-20 lipoprotein molecules in the serum,[3-6] and (4) an increase in the readily extractable lipid in serum.

It is currently assumed that phospholipid maintains a stabilizing effect upon the water-insoluble fats and sterols which circulate in the blood. These substances are transported largely in combination with proteins from the beta-globulin fraction of the plasma and are maintained in solution by the hydrophilic action of phospholipid which, through its polarity, is able to preserve the lipid-water-protein bonds. A reduction of phospholipid activity would lower the solubility by weakening the bond forces resulting in lipemia. Under these conditions, even without lipemia, cholesterol or fats are easily split from the protein carrier and may be deposited in the blood vessels.

Because of the ability of heparin to clear lipemic sera, in vivo, and to reverse the abnormal lipoprotein pattern demonstrated in atherosclerosis, it

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was important to determine whether or not these effects were accomplished by altering the rate of phospholipid synthesis. Use was made of the measurement of newly formed phospholipids following the administration of the radioactive isotope P^{32}. It has been demonstrated that the liver is the primary site of phospholipid formation and utilization. Our study was designed to observe the effects of heparin upon the rate of formation of phospholipid in control patients and in patients with coronary arteriosclerosis.

METHODS

The patients utilized for phospholipid turnover studies were hospitalized in the Temple University and Episcopal Hospitals. None of the patients in the initial study had any evidences of atherosclerosis or hepatic disease. Among this group there were ambulatory, convalescent patients, patients with psychoneurosis, and one with minimal tuberculosis. A total of eleven patients were given radioactive phosphorus, of whom five received heparin at the time of the administration of the tracer dose; the other six served as controls and received no heparin. Subsequently, four patients with typical symptoms of coronary insufficiency of several years' duration were given radioactive phosphorus together with heparin for comparison with the patients who were free of vascular disease. The dosage of heparin varied from 50 to 100 mg. given intravenously. Heparin* administration began twenty-four hours after the P^{32} had been given in order to determine whether the specific activity-time curve of phospholipid formation would be altered by this agent. The dose of P^{32} varied from 150 to 260 microcuries injected intramuscularly. The chemical form of the isotope, received from the Atomic Energy Commission, Oak Ridge, was H_3PO_4 in weak HCl, greatly diluted.

Blood was drawn from the patients at six, twelve, twenty-four, forty-eight, and seventy-two hours following administration of the radioactive phosphorus. The serum was separated immediately and stored until the last sample was collected. One cubic centimeter of each serum sample was pipetted directly into a small metal cup and evaporated to dryness in an oven at 60° C. Another 1.0 c.c. aliquot from each serum sample was pipetted into a test tube containing 3.0 c.c. of 10 per cent trichloracetic acid. The precipitate was separated by centrifugation and the supernatant poured into another test tube. The precipitate was washed twice with 1.0 c.c. portions of 5 per cent trichloracetic acid, centrifuging each time and pouring off the supernatant into the same test tube. Finally, the precipitate was dissolved with 1.0 c.c. of a 3 per cent solution of NaOH and the solution was poured into another small metal cup. The test tube containing the precipitate was washed with 1.0 c.c. of distilled water and this was added to the cup. A third metal cup was filled with a 2.0 c.c. aliquot of the total supernatant or inorganic P^{32}, the total volume of supernatant being recorded. These two samples were also evaporated to dryness.

The three metal cups for each serum sample were then counted and the results corrected for (a) self absorption of the P^{32} beta rays within the samples, (b) for total volume of supernatant, and (c) for decay; the corrected counts per minute were converted to per cent of P^{32} dose given by determining the counts per minute per microcurie under the given counting conditions. The sum of the corrected counts per minute in the protein bound and the supernatant fraction (lipid and inorganic phosphorus, respectively) should equal the corrected counts per minute in the total phosphorus or serum sample in each case. Because of experimental errors such as uneven distribution of radioactivity in the counting sample, this was not always true. Corrections were made to obtain the most reliable lipid and inorganic P^{32} values in those cases. The counting rates obtained over these samples were very low, varying from 3 counts per minute to 300 counts per minute above the background room count. Statistics were applied to determine the length of time the samples had to be counted to keep the uncertainty in the results due to the random nature of the radioactive disintegrations within the limits required for the results to be significant. The chemical determinations involved were inorganic phosphorus from the filtrate using the method of Fisk and Subbarow and lipid phosphorus in the precipitate using the method of Hack.

*Supplied by Lederle Laboratories.

RESULTS

The concentrations of phosphorus and radioactivity in the phospholipid and inorganic fractions are expressed as specific activities. The specific activity represents "units" of radioactivity per milligram of phosphorus. It has previously been determined[5, 9] that the amount of radioactive phosphorus in the inorganic fractions rises sharply after the tracer dose, attaining a peak within a few hours. As mixing occurs across vascular boundaries and equilibrium is established within extracellular fluids, this curve falls rather rapidly.

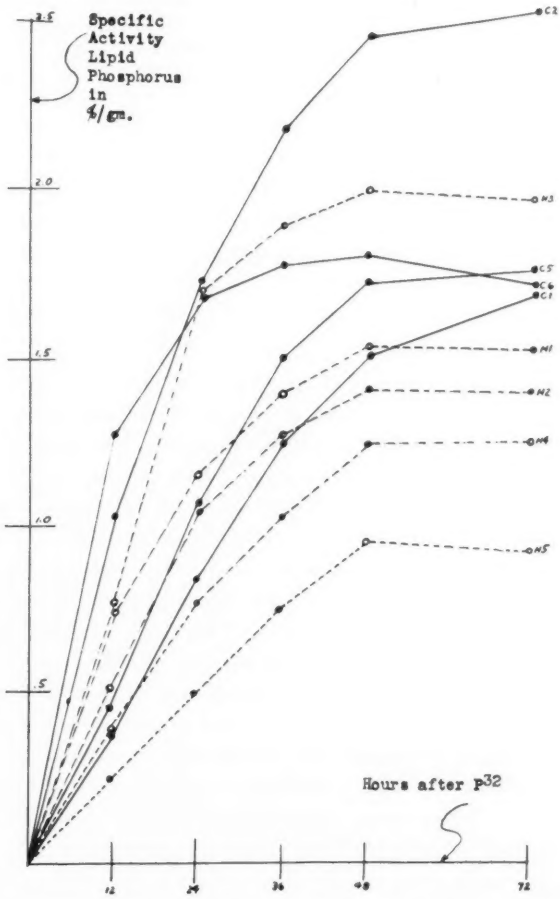

Fig. 1.—Specific activity of serum phospholipid versus time curves. Heparin was administered at twenty-four hours, the P^{32} at zero time.

In this interval the renal excretion of phosphate has a very high specific activity. This curve then flattens out slightly, this probably representing transfer into intracellular compartments. The curve then assumes a gradual slope which represents metabolic and excretory loss after complete mixing has occurred. By extrapolation back to zero time the plasma inorganic phosphorus which is initially available for the chain of synthesis leading to the formation

of phospholipid can be determined. The maximum phospholipid phosphorus specific activity can then be expressed as a multiple of the zero time inorganic phosphorus specific activity to obtain the relative specific activity, and the newly formed phospholipids at various times after the administration of P^{32}.

It is felt that the relative differences in the lipid and inorganic radioactive phosphorus levels at the various times on a single patient have been determined with great accuracy (within 5 per cent). However, no attempt was made to get exact values for the absolute level of radioactivity in percentage of dose given. To do this it would be necessary to use a small amount of carrier phosphorus in the solution injected to make sure that a minimum of the radioactive phosphorus was lost on the glass of the syringe. And it would be necessary to make a counting standard from a small aliquot of the actual solution to be injected so that the counts per minute per microcurie factor could be determined exactly for each patient studied. With the technique used, two studies on the same patient might be expected to diverge in absolute levels of radioactive phosphorus by as much as 20 per cent, although the shape of the level versus time curves should agree within 5 per cent. The chemical lipid phosphorus and inorganic phosphorus values showed remarkable constancy in both the control and heparin-treated groups throughout the period of observation.

This study confirms the previous finding that there is considerable variation in the rate of phospholipid formation in normal individuals. With such wide individual variability in the levels of phospholipid phosphorus and in the rate of phospholipid formation, it is apparent that the individual should serve as his own control to detect minor changes due to heparin effect. This procedure was not utilized because the present observations were made on essentially normal subjects whose hospitalization was brief. At the outset of this work we had anticipated showing an alteration in the specific activity versus time curves following the injection of heparin. Heparin was administered twenty-four hours after P^{32} because it was thought that a break in the curve would be produced. However, as illustrated in the graph, no significant variations in the shape of the phospholipid specific activity versus time curve in the control and heparin-treated groups could be detected. Similarly no differences were noted in the values obtained in coronary insufficiency patients treated with heparin. In order to detect changes in turnover rates for phospholipid it will be necessary to repeat this study administering heparin prior to P^{32} and obtaining more samples at earlier periods following the phosphorus injection.

DISCUSSION

The evidence that heparin could influence the state of serum lipids, leading to the present study, began to accumulate with the observations of Hahn[8] who demonstrated an increase in the translucency of alimentary lipemic plasma in vivo. Others[10, 12] have observed similar changes, and Weld[10] has shown by injecting heparinized lipemic plasma into various body regions that this clearing effect was not limited to any specific vascular bed. Heparin in its free form is not capable of inducing this clearing phenomenon and it is likely that

TABLE I. SPECIFIC ACTIVITIES OF INORGANIC AND LIPID PHOSPHORUS IN CONTROL GROUP*

PATIENT	HOURS					
	6	12	24	36	48	72
C 1	.177†	.363	.84	1.21	1.49	1.67
	13.6	7.36	4.03	3.56	2.56	2.03
C 2	5.15	1.03	1.71	2.17	2.46	2.58
	9.54	6.69	4.84		3.22	2.85
C 3	‡					
	10.1	5.50	3.98		2.31	2.20
C 4	‡					
	10.6	5.84	4.03		2.43	1.81
C 5	.20	.422	.107	.150	1.72	1.76
	8.80	5.62	3.34		2.74	2.03
C 6	.561	.126	1.68	1.181	1.83	1.70
	7.67	4.25	2.75		2.30	1.50

*Measured in per cent P^{32} per gram P^{31}.

† $\frac{\text{Lipid phosphorus}}{\text{Inorganic phosphorus}}$.

‡No chemical lipid levels determined.

the injection of heparin results in the formation of an "active principle," the latter being capable of inducing alterations in vitro. Anderson and Fawcett[11] have called this the "antichylomicronemic" factor, and have proposed that the formation of a highly surfaced active heparin-phospholipid complex was the probable mechanism of action. It has been determined by ultracentrifugal study that this "active principle" is contained in the globulin fraction of plasma[1, 14] and, when this fraction from heparinized plasma is incubated with earlier post heparin plasma, further in vitro alterations of lipoprotein distribution in such plasma are produced. Other anticoagulants, such as protamine, fast pink B.L., and sulfonated polysaccharides have also been shown to produce clearing effects similar to heparin.[12] Graham and associates,[1] by ultracentrifugal study, demonstrated profound reorientation of the distribution of low-density lipoproteins characterized by a shift of lipoproteins of high S_f

TABLE II. SPECIFIC ACTIVITIES OF INORGANIC AND LIPID PHOSPHORUS IN HEPARIN GROUP*

PATIENT	HOURS					
	6	12	24	36	48	72
H 1	.410†	.706	1.13	1.38	1.56	1.53
	8.80	5.06	2.41		1.91	1.59
H 2	.12	.398	1.05	1.26	1.40	1.41
	5.70	3.89	3.63		1.56	1.30
H 3	.483	.793	1.69	1.90	2.00	1.93
	8.08	4.48	2.77		1.57	1.51
H 4	.250	.398	.738	1.02	1.23	1.23
	8.33	4.21	2.75		1.86	1.71
H 5	.119	.262	.488	.716	.910	.905

*Measured in per cent P^{32} per gram P^{31}.

† $\frac{\text{Lipid phosphorus}}{\text{Inorganic phosphorus}}$.

classes, with maximal changes in the lower classes being observed in about three hours. Repository heparin prolonged these changes for many days.

The exact mode of action of heparin in transforming the higher S_f molecules to those of successively lower classes is not clear; however, it is postulated that there is an actual transformation of the former into the latter. This is significant because it may represent a hint to the further understanding of the serum lipoproteins, especially if this conversion is part of the normal metabolism of fat, since the latter is represented primarily in the S_f 17-100 classes of lipoproteins. Some[1] have proposed that a heparin-like substance may be involved in the physiologic interconversion of lipoproteins and that a deficiency of this "active factor" may result in abnormal elevations of the high S_f class molecules in the blood.

It would appear that no appreciable differences exist in the abilities of the control and heparin-treated patients to incorporate recently injected P^{32} into the plasma phospholipids. In the ultracentrifugation studies it was demonstrated that the administration of heparin was quickly followed by a conversion of the high S_f classes of lipoprotein to the lower S_f classes. These latter particles have a higher percentage concentration of phospholipid than do the high flotation particles,[13] but there is no evidence to suggest that the total concentration of phospholipid in the plasma was altered following the use of heparin.

It has been shown that the liver is not only the site of phospholipid formation but also is the principal organ involved in its utilization. Possibly more frequent observations on phospholipid specific activity during the first six hours following the administration of heparin and P^{32} would demonstrate a change in the rate of metabolism by the liver of this lipid fraction, since the rapid phase of the physical transition of plasma lipoproteins occurs within this interval. The present study was designed to show only gross changes in phospholipid metabolism which would have produced qualitative differences in the specific activity curves obtained after heparin administration. In the absence of such changes, it is felt that the more careful evaluation of specific activity curves in the first twelve hours should be made to provide more suitable data from which the action of heparin upon turnover rate could be studied. The present findings do not support the thought that heparin or heparin-like substances are involved in the metabolic utilization of lipoproteins. The observations of others[10, 11, 14] that the recorded effects of heparin on serum lipids and lipoproteins reflect only the physical alterations occurring in these substances, such as changes in surface tension, lipid-protein bond forces, and particle size. Anfinsen and associates[14] have shown that the reorientation phenomenon occurring in lipoproteins after heparin administration is dependent upon the presence of a clearing factor derived from the combination of heparin with a component of plasma, fraction IV-1.

CONCLUSIONS

Although heparin is capable of producing alterations in the blood lipoprotein spectrum, it did not significantly alter the specific activity-versus-time

curves of phospholipid in normal and coronary arteriosclerotic patients as measured by the radioactive phosphorus tracer technique. Measurement of the newly formed phospholipid in a group of control patients gave specific activity values which were similar over a seventy-two hour period to those obtained in heparin-treated patients. It is possible that the effects produced by heparin are of a physicochemical nature, rather than an alteration in lipid metabolism or lipoprotein interconversion. However, it will be necessary to obtain additional data relating to the turnover rate of phospholipid, by studying the specific activity curve within the first twelve hours after the injection of P^{32}.

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COLD AGGLUTINATION OF SHEEP ERYTHROCYTES AS A FACTOR IN FALSE-POSITIVE HETEROPHILE AGGLUTINATION TESTS

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In 1932, Paul and Bunnell[1] reported that serum specimens from patients with infectious mononucleosis contain agglutinins for sheep erythrocytes, often in high titer. This observation formed the basis for the so-called heterophile agglutination test for infectious mononucleosis. Although the Paul-Bunnell test has since been employed widely, it has not been entirely satisfactory in that a significant number of so-called "false-positive" reactions have been observed. It has been suggested that many of the discrepancies in heterophile agglutination results may be due to the method by which the test is performed.[2] For example, Stuart and his associates[3] have shown that the concentration of sheep cells employed in the test exerts a profound influence on the degree of agglutination. In addition, Zarafonetis and Oster[4] have demonstrated that wide variations exist in the agglutinability of erythrocytes from different sheep. The role of these elements may be controlled, however, by using a standard concentration of sheep cells in all tests,[3] and by testing all sera from a given patient against the same lot of sheep cells.[4]

Aside from variations in sheep-cell concentration and agglutinability, confusion may arise from the fact that antibodies other than those resulting from infectious mononucleosis may agglutinate sheep erythrocytes. In this connection, Davidsohn[5] has shown that the heterophilic antibodies in infectious mononucleosis are not of the Forssman type, that is, they are not completely absorbed by a suspension of guinea pig kidney. His extensive experience has led him to conclude that the heterophilic antibodies (antisheep agglutinins) in healthy persons, in serum sickness, and in all other diseases except infectious mononucleosis are of the Forssman type. Since the Forssman antibodies are absorbable by suspenions of guinea pig kidney, Davidsohn[5] has devised a differential absorption test to which doubtful sera may be subjected. This relationship of Forssman antibodies to false-positive heterophile agglutination reactions has been well documented, of course, and need not be dwelt upon here.

More recently, observations made during the course of an extensive study of the heterophile agglutination reaction indicated that cold agglutinins for sheep cells may also be present in patient's sera and give rise to false-positive Paul-Bunnell tests.[2] The phenomenon of cold agglutination was originally described by Landsteiner in 1903.[6] He described reactions between red blood cells and sera of normal guinea pigs, chickens, horses, dogs, rabbits, and cattle in which agglutination was observed at cold temperatures but disappeared on warming the tubes. Stuart and co-workers[3] were the first to call attention to the occurrence of cold agglutination in relation to the Paul-Bunnell test. They also pointed out that the sheep cell agglutination associated with serum antibody elicited by infectious mononucleosis is a "warm" agglutination, occurring and persisting at 37.5° C. Stuart and his associates, therefore, recommended that following overnight refrigeration, all heterophile agglutination tests be incubated for two hours at 37.5° C. in order to dispel any superimposed cold agglutinin

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effect. That this point is not generally appreciated, however, is evident upon a review of the literature which reveals that many laboratories record the heterophile agglutination titer after overnight refrigeration. Indeed, this step is included in the test instructions of an eminent text.[7] For this reason, it is deemed worth while to present at this time certain observations regarding cold agglutinins for sheep erythrocytes in order to emphasize their possible relationship to false-positive heterophile agglutination tests. As in a previous report,[4] the question of variation in agglutinability of erythrocytes from different sheep, as well as the testing of sera for cold agglutinins, was explored in this study.

MATERIALS AND METHODS

Source of Sera: The serum specimens employed in this study were selected from a total collection of 6,432 specimens obtained from 2,221 individuals during the past several years. The patients represent a wide range of clinical material, as has been indicated elsewhere.[2, 8, 9] For purposes of this investigation, sera from subjects with infectious mononucleosis and with conditions other than infectious mononucleosis were used. The diagnosis of infectious mononucleosis, where applicable, was established on the basis of clinical, hematologic, and heterophile agglutination findings. The noninfectious mononucleosis sera were from patients with known diagnoses.

Source of Sheep Cells: Blood samples obtained weekly from random sheep were employed in the routine tests. In addition, blood specimens were collected from each of 24 Shropshire sheep at the time of slaughter. The collections were made in 2 groups of 12 each. The specimens were defibrinated by shaking with glass beads and stored in the refrigerator at 4° C. Tests with erythrocytes from the respective sheep were begun the day after collection. For each day's test the cells were washed with physiologic saline solution and packed by centrifugation in the customary fashion. No lot of sheep cells was used longer than one week from the date of collection.

Technique of heterophile agglutination test with control for cold agglutinin effect: The technique of test employed in this laboratory has been given in detail elsewhere.[2] Briefly, the sera are inactivated at 56° C. for twenty minutes, and 0.5 ml. volumes of serial twofold dilutions of each are prepared in rows of test tubes. To each tube is then added 0.5 ml. of a 1 per cent suspension of washed sheep cells. The diluent throughout is physiologic saline solution. The sera and cells are mixed thoroughly by shaking, and then incubated in the water bath at 37.5° C. for four hours. At the end of this time a preliminary reading is made, the tubes are again shaken, and then placed in the refrigerator at 4° C. overnight. On the following morning another reading is taken immediately after removing the tubes from the refrigerator. In order to dispel any cold agglutinin effect, the test is then returned to the water bath (37.5° C.) for two hours, after which the final reading is made. Readings range from 4 plus (complete agglutination) to 0 (none). Agglutination of 2 plus is recorded when at least half of the cells in a tube is estimated to be agglutinated. Any agglutination reading of less than 2 plus is arbitrarily ignored in recording the end point titer. Titers are recorded in the final serum dilution in each instance.

RESULTS

In Table I are presented the findings in tests on two infectious mononucleosis sera with erythrocytes from 24 different sheep. It is evident that overnight refrigeration often increases the heterophile agglutination titer over that obtained after an initial incubation of the tubes for four hours at 37.5° C. It is also apparent that a significant reduction of the post-refrigeration titer may follow further incubation for two hours at 37.5° C. The range of titer change attributable to cold agglutinins was wide for both sera. It should be noted, moreover, that cells from less than half of the sheep showed agglutination reversible by rewarming of the tubes after overnight refrigeration. Similar results were obtained with 22 other infectious mononucleosis sera tested against all 24 lots of sheep cells.

TABLE I. RESULTS OF TESTS WITH ERYTHROCYTES FROM TWENTY-FOUR SHEEP AND TWO INFECTIOUS MONONUCLEOSIS SERA

CELLS FROM SHEEP NO.	TITER OF SERUM 49-386 AFTER: 4 HR. 37.5° C.	OVERNIGHT 4° C.	2 HR. 37.5° C.	CELLS FROM SHEEP NO.	TITER OF SERUM 49-466 AFTER: 4 HR. 37.5° C.	OVERNIGHT 4° C.	2 HR. 37.5° C.
1, 2.	1/64	1/128	1/128	1, 2, 20.	1/256	1/512	1/512
3.	1/64	1/512	1/256	3, 4, 7, 11,	1/256	1/1024	1/512
4, 9, 10.	1/128	1/512	1/256	5.	1/64	1/256	1/256
5.	1/32	1/128	1/64	6.	1/128	1/256	1/256
6.	1/64	1/64	1/64	8, 12, 16.			
7, 17, 21.	1/128	1/256	1/256	17, 23, 24.	1/512	1/1024	1/1024
8.	1/128	1/1024	1/256	9.	1/512	1/2048	1/512
11, 14.	1/128	1/256	1/128	10.	1/512	1/1024	1/512
12, 19, 23.	1/256	1/512	1/256	13, 15, 22.	1/1024	1/1024	1/1024
13, 15, 16, 18.				14, 19.	1/256	1/512	1/256
20, 22, 24.	1/256	1/256	1/256	18.	1/256	1/1024	1/1024
				21.	1/512	1/512	1/512
Range	1/32-1/256	1/64-1/1024	1/64-1/256		1/64-1/1024	1/256-1/2048	1/256-1/1024

The results of tests with cells from 2 sheep and 24 infectious mononucleosis sera are given in Table II. The fact that the erythrocytes as well as the sera greatly influence the agglutinin phenomenon is more sharply brought out in this table. It may be seen also that the further incubation at 37.5° C. enhanced the titer of some sera, especially in the tests with cells from sheep No. 6.

That the cold agglutinin phenomenon may be detected throughout the course of infectious mononucleosis is shown in Table III which summarizes the findings in 4 cases of this disorder.

TABLE II. RESULTS OF TESTS WITH ERYTHROCYTES FROM TWO SHEEP AND TWENTY-FOUR INFECTIOUS MONONUCLEOSIS SERA

SERUM	TITER WITH CELLS OF SHEEP NO. 3 AFTER: 4 HR. 37.5° C.	OVERNIGHT 4° C.	2 HR. 37.5° C.	TITER WITH CELLS OF SHEEP NO. 6 AFTER: 4 HR. 37.5° C.	OVERNIGHT 4° C.	2 HR. 37.5° C.
49-387	1/32	1/32	1/32	1/32	1/32	1/32
49-51	1/32	1/256	1/256	1/64	1/64	1/64
49-470	1/32	1/256	1/256	1/64	1/64	1/64
49-386	1/64	1/512	1/256	1/64	1/64	1/64
49-376	1/256	1/4096	1/2048	1/512	1/512	1/512
49-478	1/2048	1/8192	1/2048	1/512	1/2048	1/1024
49-466	1/256	1/1024	1/512	1/128	1/256	1/256
49-383	1/512	1/1024	1/1024	1/512	1/512	1/512
49-225	1/512	1/1024	1/1024	1/128	1/256	1/256
49-161	1/1024	1/2048	1/1024	1/256	1/512	1/512
49-210	1/1024	1/4096	1/1024	1/256	1/512	1/512
49-248	1/512	1/2048	1/512	1/256	1/512	1/512
48-2444	1/2048	1/4096	1/4096	1/1024	1/1024	1/2048
49-8	1/1024	1/2048	1/1024	1/512	1/512	1/1024
49-127	1/1024	1/1024	1/1024	1/512	1/512	1/512
49-140	1/512	1/512	1/512	1/256	1/256	1/512
48-2101	1/512	1/1024	1/1024	1/256	1/256	1/512
48-2197	1/256	1/256	1/512	1/128	1/256	1/256
48-1096	1/64	1/128	1/64	1/32	1/32	1/32
48-2265	1/1024	1/2048	1/2048	1/256	1/512	1/2048
49-401	1/64	1/256	1/128	1/32	1/64	1/64
49-516	1/32	1/128	1/64	1/32	1/32	1/32
48-2117	1/512	1/2048	1/2048	1/256	1/256	1/512
48-2175	1/512	1/1024	1/1024	1/256	1/256	1/256

TABLE III. RESULTS OF TESTS ON SERA FROM FOUR CASES OF INFECTIOUS MONONUCLEOSIS

CASE NO.	DAY OF DISEASE OF SERUM TESTED	TITER AFTER: OVERNIGHT 4° C.	TITER AFTER: 2 HR. 37.5° C.
376	5	1/16	1/8
	13	1/1024	1/256
	19	1/4096 NEP	1/1024
	27	1/4096	1/1024
	32	1/4096	1/512
	117	1/128	1/64
855	10	1/1024	1/512
	25	1/1024	1/512
	41	1/256	1/128
	74	1/128	1/16
	482	1/128 NEP	1/16
1319	10	1/256	1/256
	18	1/4096	1/2048
	24	1/4096	1/2048
	45	1/4096	1/1024
	60	1/1024	1/256
	126	1/512	1/128
	153	1/256	1/64
	186	1/256	1/64
1908	2	1/64	1/8
	5	1/64	1/32
	27	1/256	1/256
	40	1/256	1/128
	62	1/128	1/64
	76	1/128	1/16

NEP—No end point reached.

Table IV shows the test results in Case 583, a patient with infectious mononucleosis. It may be seen that the same sera, tested with cells from different sheep, gave widely different titers in both warm and cold agglutination.

That cold agglutination of sheep erythrocytes occurs with sera from patients with other diseases is illustrated by the results given in Table V. Although only 48 cases are listed in order to bring out this point, the number could easily be tripled from data already at hand.

TABLE IV. RESULTS OF TESTS ON SERA FROM CASE NO. 583 USING ERYTHROCYTES FROM TWO DIFFERENT SHEEP

	DAY OF DISEASE OF SERUM TESTED	TITER AFTER: OVERNIGHT 4° C.	TITER AFTER: 2 HR. 37.5° C.
Sheep A	7	1/2048	1/1024
	10	1/1024	1/256
	33	1/512	1/128
	55	1/64	1/32
	76	1/64	1/16
Sheep B	7	1/4096	1/1024
	10	1/4096 NEP	1/1024
	33	1/2048	1/256
	55	1/256	1/64
	76	1/256	1/32

NEP—No end point reached.

TABLE V. COLD AGGLUTININS FOR SHEEP ERYTHROCYTES IN TESTS WITH SERA FROM PATIENTS WITH DIAGNOSES OTHER THAN INFECTIOUS MONONUCLEOSIS

CASE NO.	DIAGNOSIS	TITER AFTER: OVERNIGHT 4° C.	TITER AFTER: 2 HR. AT 37.5° C.
42	Aplastic anemia	1/64	1/16
107	Congenital hemolytic anemia	1/64	0
1429	Acquired hemolytic anemia	1/64	0
32	Pernicious anemia	1/64	0
719	Pernicious anemia	1/64	1/16
1221	Pernicious anemia	1/128	1/16
1449	Pernicious anemia	1/64	1/8
842	Hemophilia	1/64	1/16
1723	Hemophilia	1/64	0
158, 1402	Banti's syndrome	1/128	1/32
1383	Henoch-Schoenlein purpura	1/64	1/8
372	Polycythemia	1/128	1/32
168	Chronic granulocytic leukemia	1/128	1/64
335	Chronic granulocytic leukemia	1/128	1/32
340	Chronic granulocytic leukemia	1/128	1/8
882	Chronic granulocytic leukemia	1/256	0
879, 1712	Subleukemic leukemia	1/128	1/16
1863	Chronic lymphocytic leukemia	1/64	0
1897	Acute lymphoblastic leukemia	1/64	0
1334	Lymphosarcoma	1/128	1/64
1418	Lymphosarcoma	1/128	0
856	Hodgkins' disease	1/128	1/32
1402	Hodgkins' disease	1/128	1/16
1627	Dermatitis herpetiformis	1/64	0
1985	Dermatitis herpetiformis	1/64	1/16
1990	Dermatitis herpetiformis	1/128	1/32
1205	Scleroderma	1/128	1/16
1208	Scleroderma	1/64	1/8
1673	Scleroderma	1/64	1/32
1668	Dermatomyositis	1/64	1/32
1861	Dermatomyositis	1/64	1/8
733, 1116, 1231, 1652	Lupus erythematosus	1/64	0
1721	Lupus erythematosus	1/64	1/32
426	Influenza	1/128	0
532	Miliary tuberculosis	1/128	0
1125	Renal tuberculosis	1/128	1/32
356	Tuberculous meningitis	1/128	1/8
548	Tuberculous sinus tracts	1/128	1/16
549	Pulmonary tuberculosis	1/128	1/16
646	Pulmonary tuberculosis	1/128	1/8
798	Blastomycosis	1/64	1/16
1981	Amebiasis	1/64	0
242	Urinary tract infection (*A. aerogenes*)	1/128	1/16

DISCUSSION

The heterophile agglutination test of Paul and Bunnell[1] was performed by incubating the tests tubes in the water bath at 38° C. for one hour followed by overnight refrigeration in the icebox. The test results were read on the following morning immediately after the tubes were removed from the icebox. From the results obtained in this study, it is readily apparent that the phenomenon of cold agglutination may greatly affect the results of heterophile agglutination tests performed in this general manner. Furthermore, it is evident that erythrocytes from different sheep vary not only in their agglutinability at 37.5° C.,[4] but also in their response to cold agglutinins. It is of interest that the range of titer change attributable to cold agglutinins is even greater than that due to the

heterophile agglutinins of infectious mononucleosis. For example, the heterophile agglutination titer of serum 49-386 ranged from 1:64 to 1:256 in tests against erythrocytes from 24 different sheep, while serum 49-466 ranged from 1:256 to 1:1024 in tests with the same cells (Table I). The range of readings of these same sera after the refrigeration phase of the test was 1:64 to 1:1024 and 1:256 to 1:2048, respectively. Even greater differences in titer are shown in Tables II and III which give the findings in similar tests with other sera and cells.

Thus, not only may cold agglutinins for sheep cells be present in sera from patients with infectious mononucleosis, there may also be a wide divergence of titer depending on the sheep erythrocytes themselves. For example, serum 49-386 showed the cold agglutination phenomenon with erythrocytes of only 11 of 24 sheep, and serum 49-466 with cells of only 9 of 24 sheep. In this connection, the results given in Table II are of interest. Erythrocytes from 2 sheep were tested against 24 different infectious mononucleosis sera. Tests with the cells from sheep No. 3 gave cold agglutination with 11 of the sera, while only 1 of the 24 serum specimens showed cold agglutinins with erythrocytes from sheep No. 6. Indeed, further incubation at 37.5° C. increased the titer of several sera, indicating that some cell-serum mixtures require more time than others for complete antigen-antibody interaction.

The presence of cold agglutinins for sheep cells in sera from patients with other diseases is shown by the data given in Table V. Included are serum specimens from patients with leukemia, lymphoma, various anemias, collagen disorders, bullous eruptions, and acute and chronic infections which have exhibited cold agglutination in dilutions up to 1:256 in tests with random lots of sheep cells. While these titers are not as high as those observed with many of the infectious mononucleosis sera, they are of sufficient magnitude to be misleading if their true nature is not recognized. Furthermore, the dependence of titer on both the specific serum and the particular lot of sheep cells used, as shown in this study, suggests that broader laboratory experience would occasionally encounter cold agglutinin titers higher than those given herein. On the basis of the present findings alone, however, it seems reasonable to conclude that many of the "false-positive heterophile agglutination" results recorded in the literature are due to cold agglutination which has not been dissipated by the test procedure employed.

In addition to the implications of these findings with reference to sheep cell agglutination by sera from patients with infectious mononucleosis and other diseases, it seems likely that variability of the cold agglutination phenomenon pertains in other situations. For example, studies underway in this laboratory indicate that much of the confusion regarding cold agglutinins in human sera for washed type "O" cells may be due to a similar variability in agglutinability of erythrocytes from different donors.

SUMMARY AND CONCLUSIONS

(1) Cold agglutinins for sheep erythrocytes may be present in sera from patients with infectious mononucleosis and other disorders. (2) The cold ag-

glutinin titer for a given serum specimen may vary widely in tests with erythrocytes from different sheep. (3) As a corollary, erythrocytes from one sheep may give high cold agglutinin titers with the test sera, while cells from another sheep may agglutinate poorly or not at all in similar tests with the same sera. (4) Since many laboratories record titers of heterophile agglutination tests directly after overnight refrigeration of the test, it seems reasonable to conclude that many so-called "false-positive heterophile agglutination" results are due to the phenomenon of cold agglutination. (5) It is possible to eliminate the cold agglutinin factor by the simple expedient of rewarming the test serum-cell mixtures following overnight refrigeration.

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STRONTIUM SULFIDE DEPILATION OF RAT SKIN AND THE EFFECT OF LANOLIN ON DEPILATED SKIN

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NUMEROUS techniques have been devised for preparing the skin of small laboratory animals for physiological or biochemical studies. Clipping, razor, and electric shaving, stripping with adhesive tape, and chemical depilation have been used. When it became necessary in this laboratory to depilate relatively large numbers of rats, each of these techniques was tried. If electric clippers were used, little or no damage was incurred by the skin, but a fine stubble of hair remained. If in addition to clipping, electric or razor shaving was added, a surface nearly free of visible hair resulted, but within a day or two numerous serous crusts appeared on the skin. Adhesive tape stripping as used by Szczesniak and associates[2] was obviously unsatisfactory, since many small areas of bleeding were seen immediately following depilation. Chemical depilation with strontium sulfide was next attempted and, although a few serous crusts again developed after twenty-four to forty-eight hours, the result appeared more promising than with the other procedures. Further, it was found that application of lanolin following the sulfide depilation prevented the roughening and serous encrustation. Accordingly, a depilation technique making use of strontium sulfide and lanolin was developed. To evaluate the tissue injury produced by the depilation and the part played by lanolin in its prevention, the following study was undertaken.

METHODS

Female Sprague-Dawley rats (50 to 60 days old)* were lightly anesthetized with Nembutal and after electric clipping a thick paste of commercial strontium sulfide depilatory was applied to one or both flank or hip areas. After four to five minutes, the paste was washed off with a stream of cool water. The area was then rinsed with 0.1 per cent acetic acid† and finally with more cool water. Lanolin was softened by warming and then applied liberally to the depilated area one hour, two days, and four days after depilation.

Forty-two rats were clipped and depilated as described. To a group of 21 of these animals lanolin was applied one hour, two days, and four days after depilation. No lanolin was applied to the remaining group of 21. Immediately after depilation and at one, two,

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*The age of the rat at depilation was found to bear a critical relationship to the completeness of depilation. Animals younger than about 45 days, or older than 60 days, did not provide a satisfactory preparation. Rats less than 45 days old had a spotty surface with scattered areas of unremoved fine hairs; those older than 60 days had a heavy bristle which frequently was not completely removed. Results with male animals were less satisfactory than with females of the same age.

†Subsequently, observations based on gross appearance have indicated that when copious quantities of rinse water are used the acetic acid may be dispensed with.

four, seven, eight, and ten days three animals from each group were sacrificed and a portion of the depilated area removed for histologic study. A control group of three animals was clipped but received no further treatment. These animals were sacrificed immediately and skin specimens were fixed in Bouin's solution, imbedded, sectioned at 7 micra and stained with hematoxylin and eosin.

RESULTS

The gross changes seen in the depilated area are described in Table I.

TABLE I. GROSS SKIN CHANGES RESULTING FROM APPLICATION OF LANOLIN TO CLIPPED AND DEPILATED (SRS) RAT SKIN

TIME AFTER DEPILATION (DAYS)	GROSS CHANGES	
	LANOLIN TREATED	NO TREATMENT
Immediate	Hair absent	Hair absent
1	No change	No change
2	No change	Multiple, tiny, serous crusts
4	No change	Larger crusts
7	No change	Crusts dropping off
8	No change	Crusts absent
10	Slight hair growth	Slight hair growth

Microscopically, in the clipped controls, the stratum corneum had a superficial, spongy appearing layer and a deeper compact eosinophilic portion. The stratum Malpighii was normally about two cells in thickness. The deeper cells were roughly cuboidal with ovoid to round nuclei, while those more superficial were flat, had no distinguishable nuclei and contained variable amounts of a dark staining granular substance. The dermis was a loose, eosinophilic connective tissue matrix dotted with hair follicles and sebaceous glands. A very few small blood vessels were recognizable in the superficial layers of the dermis. The base of the hair follicles and some larger blood vessels were seen in the subcutaneous tissue.

Immediately after depilation: The spongy portion of the stratum corneum was absent. Neither increased vascularization nor other evidence of inflammation was present. The hair shafts were not present above the surface of the skin and in the upper half of the dermis were shriveled and dark staining. Degenerative changes of hair shafts sporadically occurred near the surface of the dermis up to four days whether lanolin was or was not applied.

Clipped and Depilated Rat Skin.—

(1) *One day:* Of the stratum corneum only the compact base layer remained and this was easily detachable from the stratum Malpighii. Cells of the stratum Malpighii were swollen and pale staining, and the entire stratum separated readily from the dermis. The superficial levels of the dermis were highly vascular.

(2) *Two days:* The superficial cells of the stratum Malpighii exhibited a marked increase in black staining granules and the deeper cells showed swelling, vacuolization and chromatin clumping. The dermis was highly vascular. Occasional small spots of a serous material were present on the surface.

(3) *Four days:* (Fig. 1.) There were numerous small areas where the epidermis was replaced by pockets of clotted serum and pus cells. The remain-

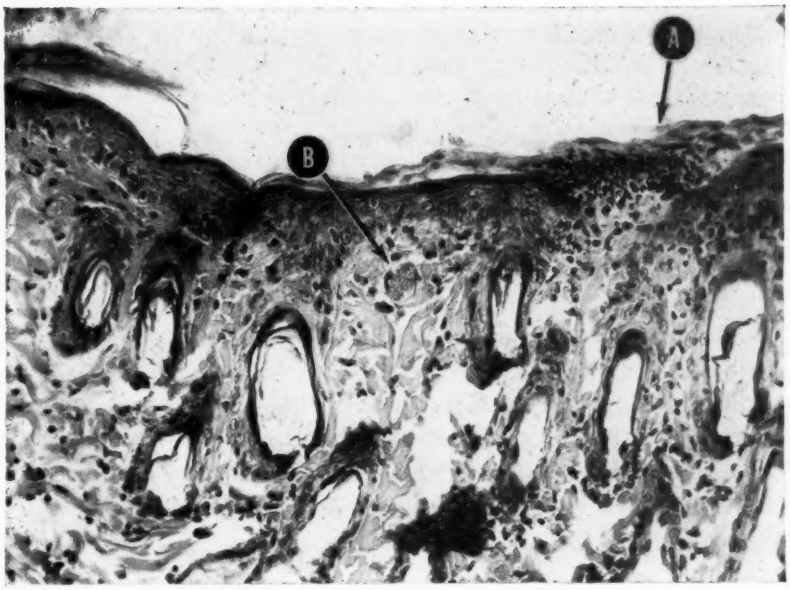

Fig. 1.—Rat skin four days after depilation with strontium sulfide. The epidermis is thickened and stratified and in areas, as at *A*, is destroyed and replaced by a small pocket of inflammatory cells and necrotic material. At *B* there is a dilated thin walled blood vessel in the superficial dermis. Degenerative changes may be seen in the hair shafts. (Hematoxylin and eosin, ×300.)

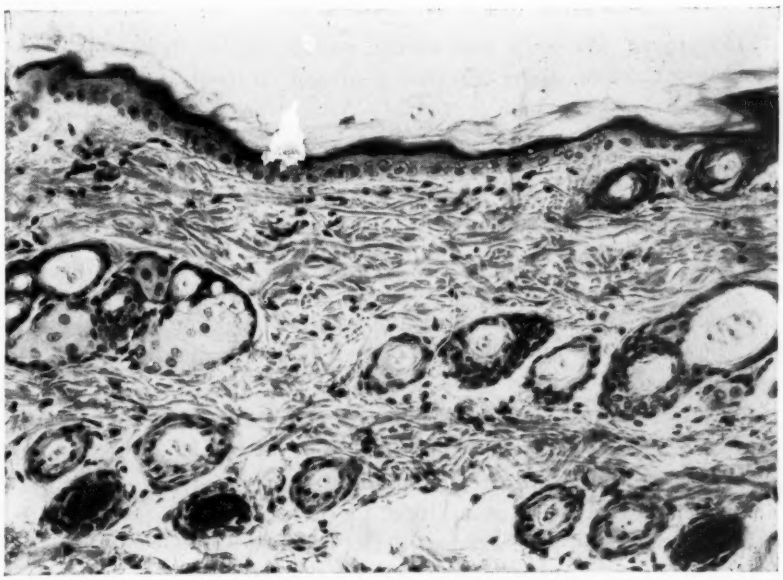

Fig. 2.—Rat skin four days after depilation with strontium sulfide. Lanolin was applied one hour and two days after depilation. Note the thin uniform epidermis. No dilated vessels were found in the superficial dermis and there were no inflammatory cells in the epidermis. (Hematoxylin and eosin, ×300.)

ing stratum Malpighii was three to five times normal thickness and stratified. The basal cells were roughly cuboidal while the more superficial ones were first polygonal and then flattened. Occasional small dilated blood vessels were present in the superficial dermis.

(4) *Seven and eight days:* Pus cells were present in the corneum. The stratum Malpighii was still stratified, but was only two to three times normal thickness. Beginning regrowth of hair shafts was evident at the surface of the dermis.

(5) *Ten days:* The spongy layer of the corneum in general appeared essentially normal. The stratum Malpighii was about twice normal thickness and the cells above the basal layer were flattened, had lost most of their nuclei and contained a large amount of the black staining substance. The vessels of the superficial dermis were considerably less prominent than at seven and eight days. The new hair shafts had penetrated the surface of the stratum corneum.

Clipped, Depilated and Lanolin Treated Rat Skin.—

(1) *One day after depilation:* The spongy layer of the corneum was absent and the stratum Malpighii separated easily from the dermis; otherwise, the skin appeared normal.

(2) *Two days:* Essentially normal.

(3) *Four days:* (Fig. 2.) Normal except for slight thickening of the stratum Malpighii in some areas.

(4) *Seven days:* A moderate increase in dark staining granular material was seen. There was a slight thickening, in a few areas, of the stratum Malpighii. The hair shafts had reached the stratum corneum.

(5) *Ten days:* The skin was normal except for an increase in the dark staining material and a slight thickening of the stratum Malpighii.

DISCUSSION

Following clipping and strontium sulfide depilation of rat skin, several changes in the skin occurred. Initially there was a removal of the hair shafts to a point below the epidermis and a loss of the superficial spongy appearing layer of the stratum corneum. After forty-eight hours a serous crust appeared on the surface and a mild inflammatory reaction was present in the epidermis and upper dermis. By ten days the inflammatory reaction had essentially disappeared; however, the epidermis remained twice normal thickness and contained a considerable excess of a black staining granular material. At ten days the new growth of hair shafts had penetrated the surface of the epidermis.

When lanolin applications were used during the first four days after depilation, the serous exudation and the inflammation of the dermis were prevented. At seven to ten days there was a slight increase in the thickness of the Malpighian layer as well as an increase in the number of dark staining granules therein; but to a much lesser extent than when no lanolin was used. Butcher,[4] on application of wool fat to clipped rat skin, found little alteration in the epidermis; in fact the wool fat appeared to maintain the epidermis in normal

condition. It is possible that the minimal changes which were seen in the present lanolin treated skins might have been prevented by continuing the lanolin application beyond four days.

The delay in appearance of the inflammatory reaction in the non-lanolin treated skins suggests that the reaction may have been the result of exposure of the skin to drying and other external factors rather than a direct result of the depilation procedure. Whether the pathology found in the strontium sulfide depilated skin is due to the action of the depilatory agent or to subsequent factors occurring in the denuded skin, lanolin effectively prevented the development of the major portion of the pathology seen in the non-lanolin treated skin.

SUMMARY

A method for the preparation of rat skin essentially free of hair has been developed. The method consists of clipping, strontium sulfide depilation, and lanolin application. Depilation without subsequent use of lanolin resulted in encrustation and inflammation at forty-eight hours, and a considerably thickened epidermis at ten days. Periodic application of lanolin following depilation essentially eliminated the pathologic changes. With lanolin only a slight thickening and an increase in granular material of the epidermis occurred.

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THE TREATMENT OF THE ACUTE RADIATION SYNDROME IN DOGS WITH AUREOMYCIN AND WHOLE BLOOD

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INTRODUCTION

ACUTE radiation sickness consequent to total body exposure is commonly characterized by hemorrhage and infection. Previous studies have at times yielded good results when experimental animals were irradiated and treated with antibiotics. Miller et al[1-3] showed that the mortality of irradiated mice could be lowered by the prophylactic administration of streptomycin. This beneficial effect was less marked if the mice developed infections caused by *Salmonella typhimurium, Pseudomonas aeruginosa,* or Proteus during the radiation sickness. Aureomycin was less effective than streptomycin. W. W. Smith and her co-workers[4, 5] tested the effects of a variety of antibiotics in irradiated mice and rats and found that the efficacy varied from group to group, possibly from species to species, and also depended on the bacterial environment of the animals. Aureomycin lengthened the survival time of rats, but did not affect the total mortality. Furth, Coulter, and Howland[6] observed no reduction of mortality when irradiated rats were treated with Aureomycin. Speck, Jawitz, and Kaplan[7] irradiated mice and then inoculated them intramuscularly with streptococcus one to three days after the radiation exposure. The infection caused the duration of survival to diminish and the total mortality to increase. The administration of penicillin reduced the mortality to the level of the uninfected irradiated controls.

Because of species differences extrapolation of these experimental data from rats and mice to dogs may be unwarranted. The use of Aureomycin for the acute radiation sickness of the dog has been tested on small numbers of animals by Furth, Coulter, and Howland,[8] Allen and associates,[9] and McDonnel and Maxwell,[10] and the results obtained suggested that some decrease in mortality might occur through the use of this antibiotic.

Prior to the experiment reported here, Allen and collaborators[9] found that blood transfusion did not affect the mortality of dogs which had received an LD_{100} of total body x-irradiation. Since our work was completed, Allen and his co-workers[11] have shown that dogs treated by transfusion of whole blood after various doses of total body radiation were not benefited by this therapy.

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METHODS

Since whole blood and antibiotics are easily available therapeutic agents which might have a beneficial effect on these major derangements, these two substances were tested alone and in combination on 192 x-irradiated dogs.

The experiment was designed to make the therapy practical. Problems of supply and administration which would arise in a military or civilian disaster were taken into consideration. Because of the difficulties in obtaining whole blood after a disaster, it was decided to use blood which had been stored a minimum of forty-eight hours. Aureomycin was chosen as the antibiotic because of its availability, ease of administration, low toxicity, and its wide range of effectiveness against infectious agents. The x-ray factors used produced a mortality of 62 and 88 per cent, so that if a reduction in the lethality brought about by the therapy occurred, it would be clearly demonstrated.

PART A

The dogs used were mongrels in which a beagle strain predominated. Their modal age was 15 to 18 months, and their modal weight was 8 to 10 kg. Dogs whose hematocrits were below 40 mm. or whose leukocyte counts were outside the range of 6,000 to 18,000 were not used. The animals had been vaccinated against distemper, dewormed, and held for observation for four to six weeks before irradiation. They were housed in individual cages and fed Purina Dog Chow Kibbled Meal.

Every two weeks a new group of 24 dogs was x-irradiated. Six dogs of each of four radiation groups were assigned to a treatment group so that when a total of 96 animals (38 female and 58 male) had been studied, each of the following treatment groups contained 24 dogs:

Group I—Controls. No therapy.

Group II—Aureomycin treated. Beginning twenty-four hours after the irradiation, one 250 mg. capsule imbedded in ground horsemeat was fed every twelve hours (50 mg. per kilogram of body weight per day).

Group III—Transfusion of stored whole blood. 125 ml. given in a ten to fifteen minute period of time whenever the dog's hematocrit fell below 80 per cent of the preradiation value. If the hematocrit had fallen to a level 70 per cent below the preradiation value, a second transfusion was given four to six hours after the first one.

Group IV—Aureomycin plus whole blood transfusion. Factors as in Groups II and III above.

The observation period was for thirty days following the x-irradiation exposure.

Hematology.—On three occasions during the week prior to irradiation, hematocrits, leukocyte counts, and platelet counts were done on blood obtained from the external jugular vein. Following irradiation these studies were performed twice weekly. In addition, hematocrit values were determined daily from the tenth to the twenty-fifth days.

TABLE I. LOWEST HEMATOCRIT PERCENTAGE OF SURVIVING DOGS

DOG NO.	GROUP	DAY POST-RADIATION	HEMATOCRIT
1870	Control	28	40
1918	Control	17	62
1944	Control	22	42
1917	Aureomycin	22	43
1923	Aureomycin	25	28
1977	Blood	14	73
1729	Blood and Aureomycin	15	62
1855	Blood and Aureomycin	20	76
1992	Blood and Aureomycin	20	71
1959	Blood and Aureomycin	28	81

Blood Donation.—A donor colony of large dogs was maintained. Donors and recipients were typed for the canine "A" factor.[12, 13] Donors which had naturally occurring isoantibodies were not used. "A" negative blood was given either to "A" positive or "A" negative recipients, but "A" positive blood was given only to "A" positive recipients. In addition major and minor cross matchings were done before each transfusion.

Two-hundred and fifty milliliters of blood were drawn from a donor into a chilled Baxter vacuum bottle containing 40 ml. of ACD solution, and stored for at least forty-eight hours at 6 to 10° C. until used. Blood stored for longer than fifteen days was not used. The donor's hematocrit values were maintained above 40 mm. by feeding each of the animals one-half pound of horse meat and 5 mg. of ferrous sulfate daily.

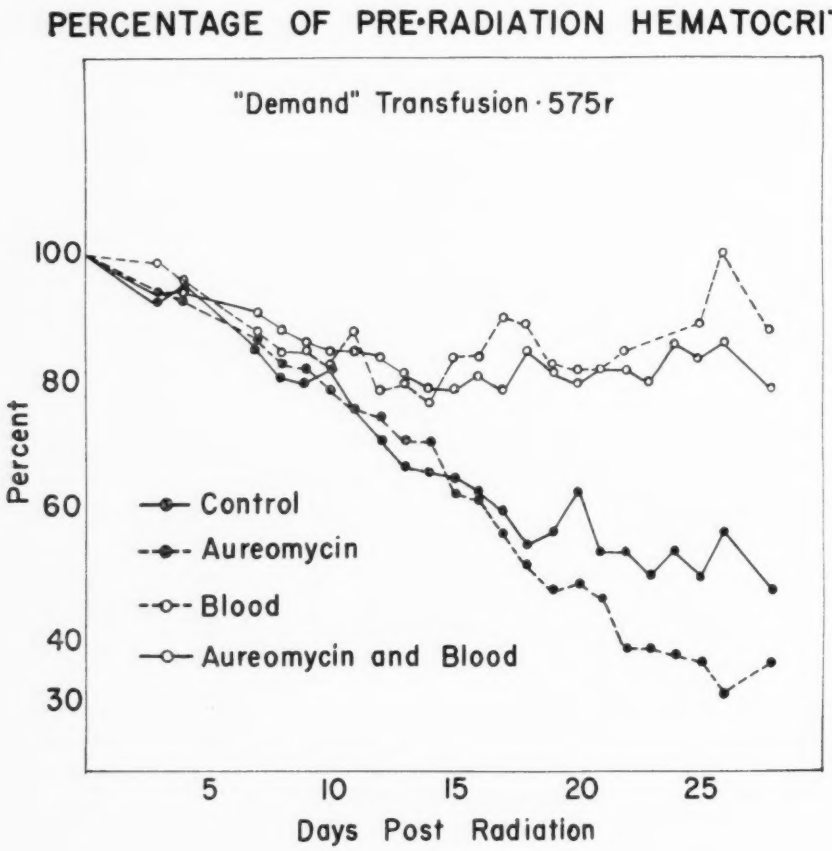

Fig. 1.

X-Ray Factors.—Dose: 575 r, 1000 k.v. General Electric Industrial x-ray unit. Lead plano convex filter one-half inch at center and one-fourth inch at the periphery. Ma: 3, rate: 8.2-8.5 r per minute, TSD: 35 inches. The dogs were irradiated while enclosed individually in wooden boxes with wire screen tops.

OBSERVATIONS

Hematology.—Fig. 1 shows that the average hematocrits of the blood and the aureomycin-blood groups remained above 80 per cent throughout the post-radiation period. The hematocrit values of the two groups which did not

receive transfusions displayed a linear decline until they were 35 to 50 per cent of the preradiation value. The lowest hematocrit percentage of surviving dogs is shown in Table I. It can be seen that an equal number of the non-transfused animals survived (lowest hematocrit values of 28 to 62 per cent) as the transfused animals (lowest hematocrit values 62 to 81 per cent).

The fall in leukocyte counts was almost identical in all four groups (Fig. 2). This was true also for the platelet determinations (Fig. 3).

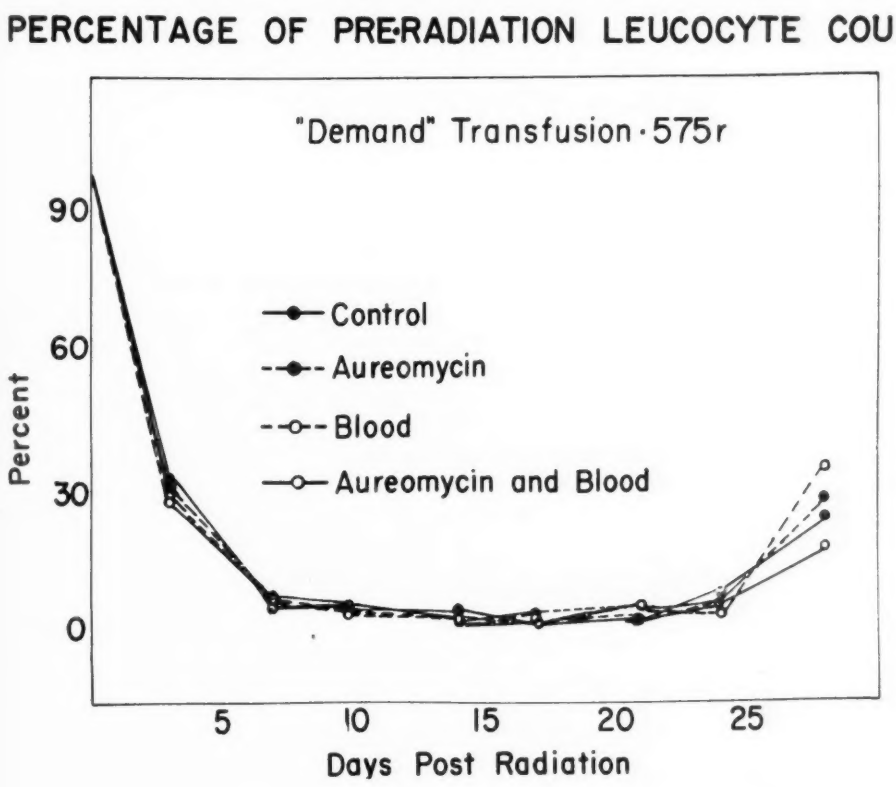

Fig. 2.

Transfusions.—140 transfusions were given to 40 dogs during the experiment. (Seven animals died before developing a hematocrit of less than 80 per cent and one survived without falling below this level, thus accounting for the other 8 eligible recipients.) No hemolytic or anaphylactoid reactions were noted, nor were there any clinical signs that the circulation had been overloaded. However, extensive edema and hemorrhage about the site of venipuncture for the transfusions were occasionally seen.

Other Clinical Signs.—Increased anorexia, vomiting, or diarrhea was not observed in the Aureomycin treated dogs as contrasted with those that did not receive this drug.

Mortality.—Fig. 4 shows the cumulative mortality for the four groups. The total deaths for the twenty-eight day period were: controls, 88 per cent; Aureomycin treated, 92 per cent; Aureomycin treated and transfused, 83 per cent; and transfused, 96 per cent.

Pathology.—Autopsies were performed and all organs except the brain were examined grossly. No significant difference in the pathology was found among the animals in the four treatment groups.

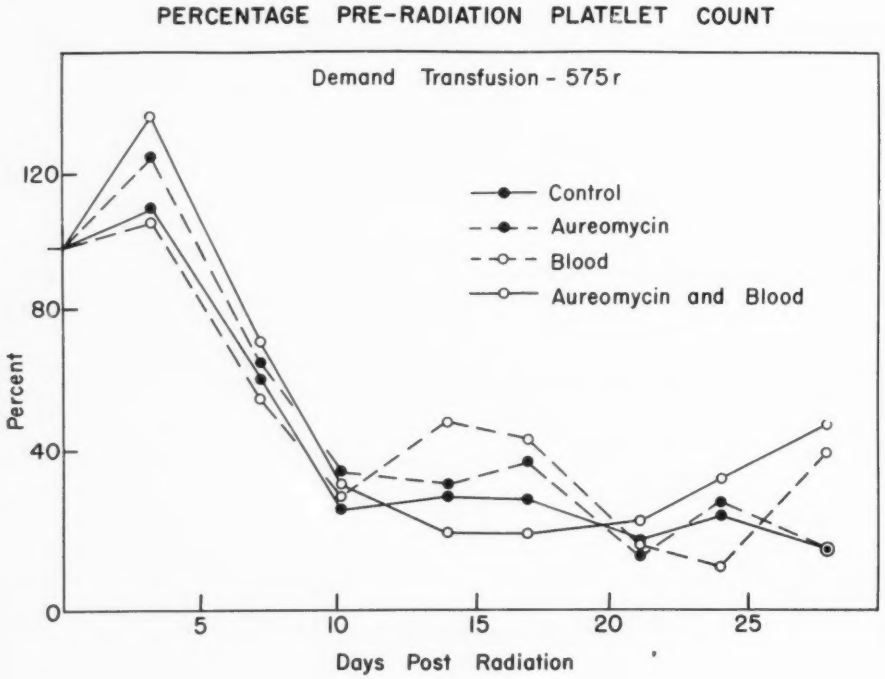

Fig. 3.

PART B

Ninety-six dogs (40 females and 56 males) were used. They were mongrels with approximately the same modal age and weight as in Part A. They were housed, fed, and grouped as described in Part A. The treatment was as follows:

Group I—Control. No therapy.

Group II—Aureomycin treated. Starting twenty-four hours after irradiation, a 250 mg. capsule of Aureomycin imbedded in a ball of ground horse-meat was fed every eight hours (75 mg. per Kilogram of body weight per day).

Group III—Transfusion of whole blood. 125 ml. given as described in Part A on the seventh, eleventh, fifteenth, and nineteenth days post irradiation, regardless of the dog's hematocrit level.

Group IV—Aureomycin and whole blood transfusion as described under Groups II and III immediately above.

Hematology.—As in Part A.

Blood Donation.—As in Part A except that the blood was stored for only forty-eight to seventy-two hours.

X-ray Factors.—Dose, 525 r; rate, 7.9 to 8.4 r per minute. All other factors as in Part A.

OBSERVATIONS

Hematology.—The hematocrits of all groups fell equally until the transfusions began. Then the blood-treated animals regained their preradiation hematocrit values while the nontransfused animals' values continued to fall (Fig. 5) . . . in the Aureomycin dogs to a lesser extent than in the control dogs. Fig. 6 and 7 show that the fall in white blood cells and in platelets were similar to those described in Part A.

Transfusions.—154 transfusions were administered to 48 dogs. No allergic or hemolytic reactions occurred.

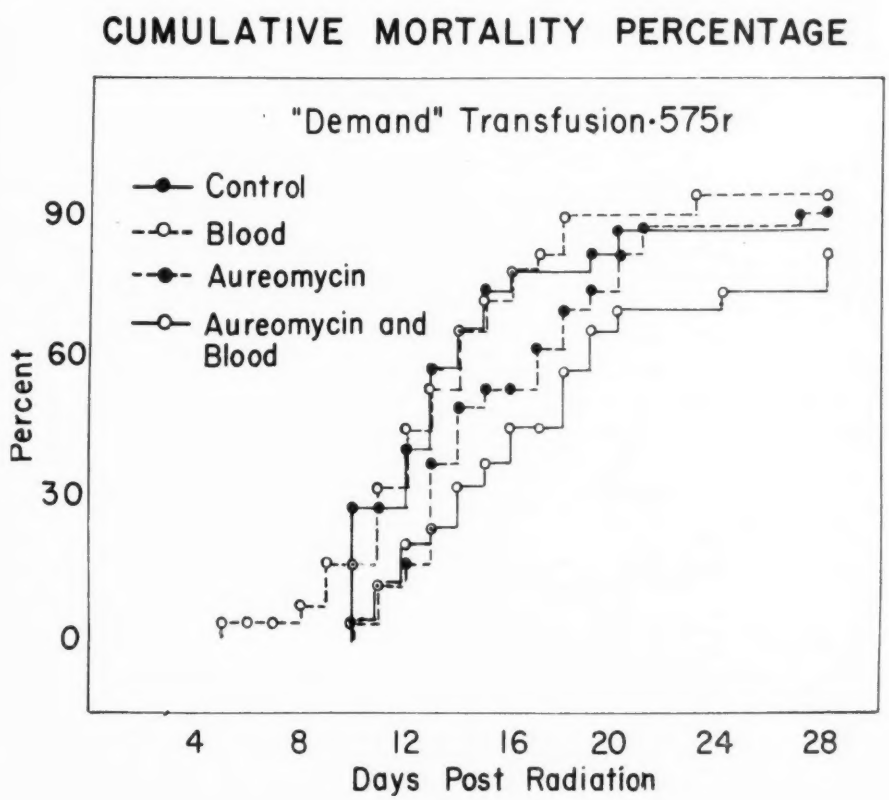

Fig. 4.

Mortality.—This is shown in Fig. 8. The thirty day mortality was: Controls 62 per cent, Aureomycin treated group 75 per cent, transfused group 79 per cent, and Aureomycin treated and transfused group 46 per cent.

Pathology.—No significant difference in the gross pathology was noted among the four groups.

DISCUSSION

Preliminary studies of the effect of Aureomycin on the acute radiation syndrome in dogs indicated that this drug diminished the mortality.[8-10] The present study using the LD_{88} dose did not confirm this earlier work. Some

of the reasons for this difference may be: (a) The radiation dose was too high, and the dogs died of hemorrhage and "cytochemical changes" even though the infection was controlled. It may be that infection plays only a small part in the radiation sickness of the dog at this dose level. (b) The Aureomycin dose was too small. (c) Organisms resistant to Aureomycin may have developed. Blood cultures were not done on these animals, but a previous study showed that among the fatalities the incidence of positive cultures was the same in the control and the Aureomycin treated animals.[14]

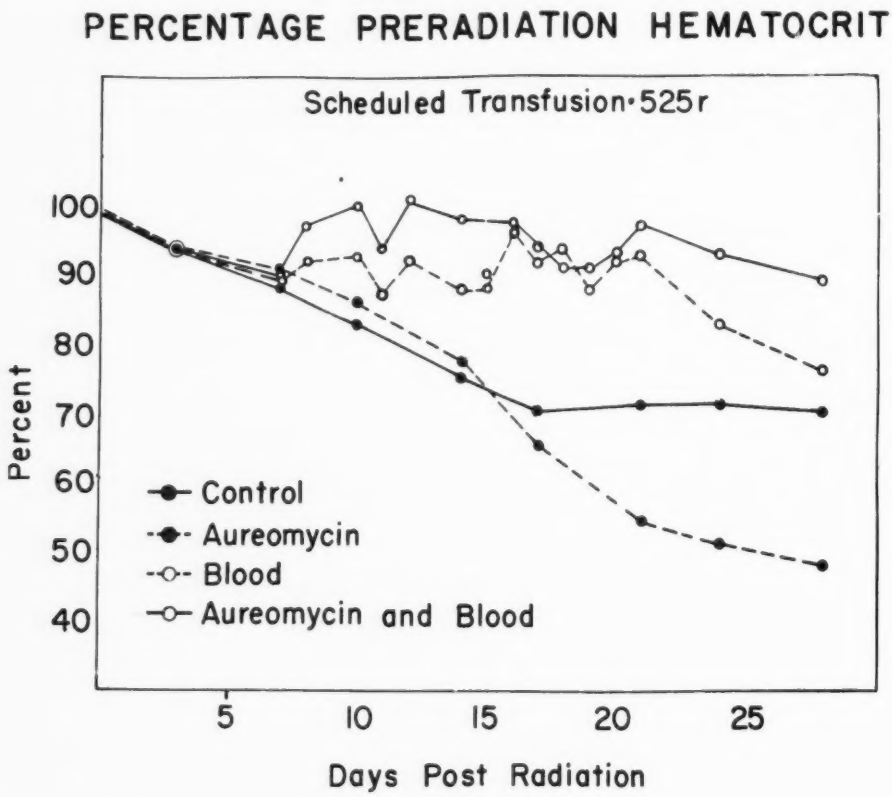

Fig. 5.

Because of the absence of leukocytes, suppurative processes cannot be readily recognized by the gross pathology of the radiated dog. Therefore, an infection other than sepsis may have been present but unrecognized.

For Part B then, the radiation dose was lowered to an LD_{62}, the Aureomycin dose was raised, and some of the variability involved in the administration of the whole blood was eliminated by transfusing the animals on a predetermined rather than a demand basis.

The failure to alter the course of the disease again in Part B suggests the presence of an Aureomycin resistant infection other than sepsis, or that infection played no part in the animal's demise, and that the transfusion of stored whole blood was ineffective.

No clinical or pathologic evidence was found which implied that the therapy was detrimental to the animals. The possibility that the transfusions overloaded the circulation was considered, but neither the autopsy findings nor the time of death of the animals confirmed this.

Our results with transfusions agree with those of Allen and co-workers[11] insofar as both indicate no beneficial results. The data of these authors, however, suggest that transfusions increased the lethality of a maximal sublethal (LD_0) dose of total body x-irradiation. We were unable to confirm this finding in our laboratory.[15]

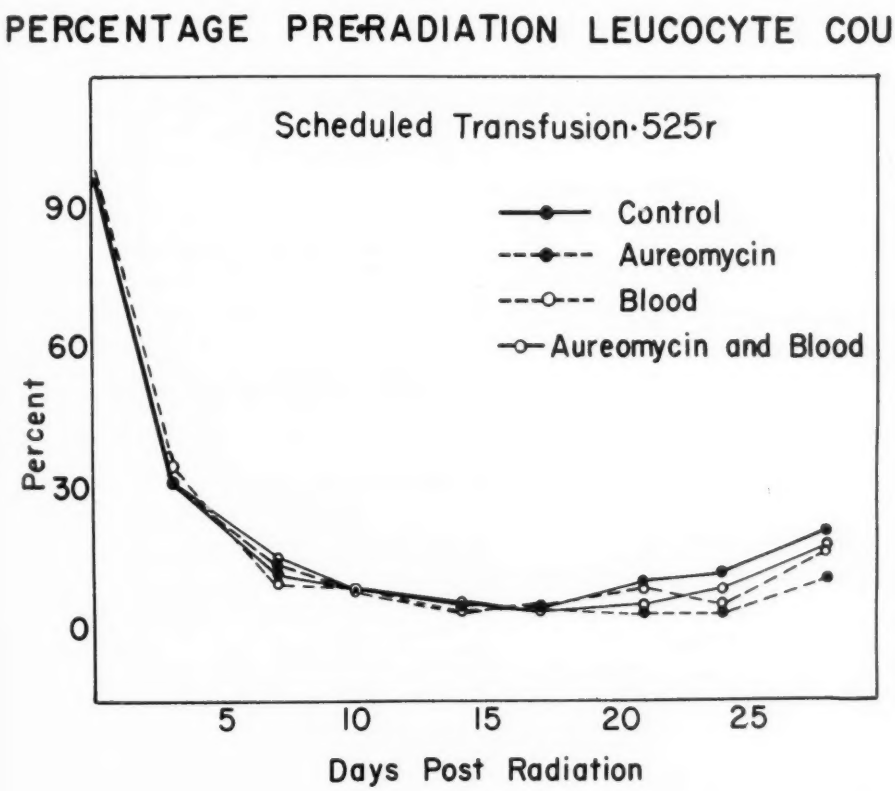

Fig. 6.

In the experiment reported here, the animals were not exposed to the infection that would be expected to threaten the human casualty of ionizing radiation. There were no epidemics of infectious disease, no potentially contaminated wounds or burns, perhaps no latent foci of infection within the animal's body. Since the irradiated subject is more susceptible to infection,[1, 2, 16] it would still seem advisable to give an antibiotic prophylactically to the victim of an atomic catastrophe.

Stored whole blood, however, would seem to have no effect other than to increase the blood volume, the hematocrit, and the plasma protein. For the patient whose life appears to depend specifically on such replacement therapy,

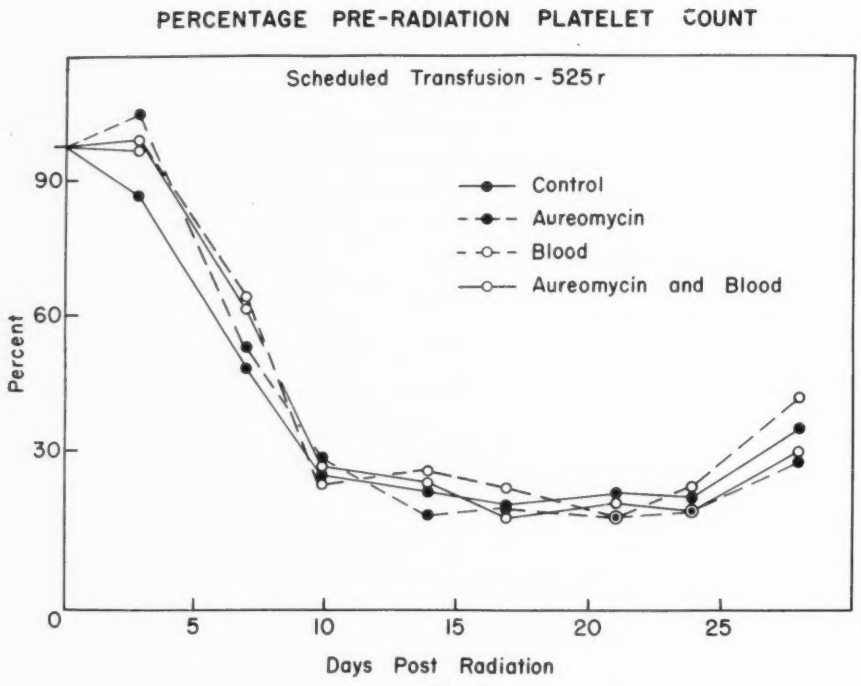
PERCENTAGE PRE-RADIATION PLATELET COUNT
Scheduled Transfusion - 525r
Control
Aureomycin
Blood
Aureomycin and Blood
Percent
90
60
30
0
5
10
15
20
25
Days Post Radiation

Fig. 7.

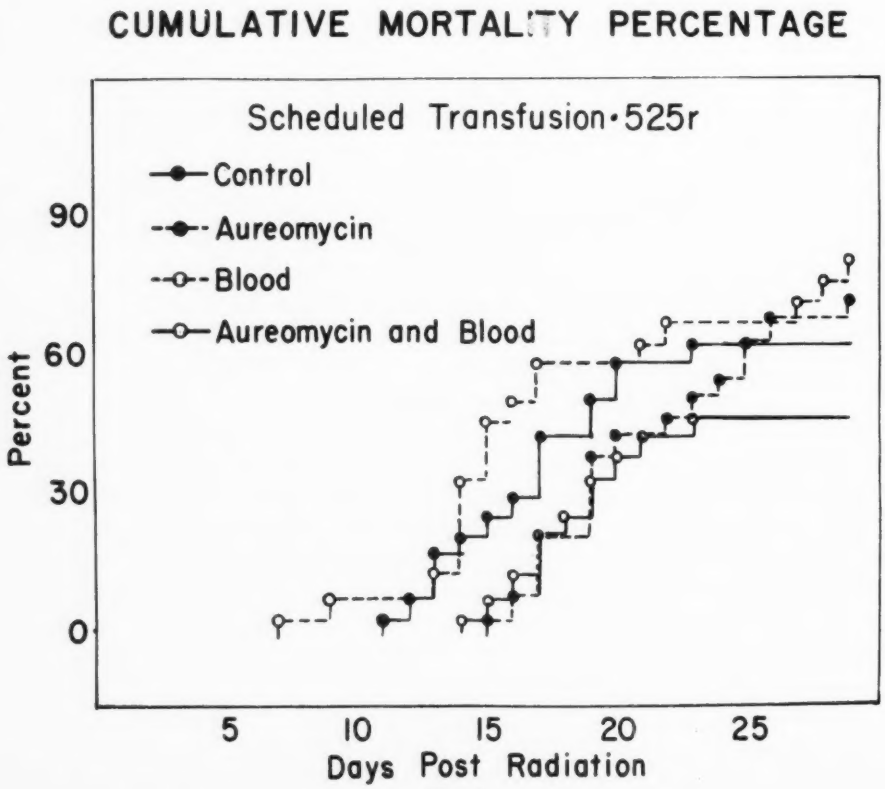
CUMULATIVE MORTALITY PERCENTAGE
Scheduled Transfusion·525r
Control
Aureomycin
Blood
Aureomycin and Blood
Percent
90
60
30
0
5
10
15
20
25
Days Post Radiation

Fig. 8.

transfusions should be administered. Transfusions of stored whole blood given prophylactically will not help to prevent the hemorrhagic diathesis.

The mortality for the control dogs (62 per cent) and those treated with Aureomycin and transfused (46 per cent) would at first glance appear to show a significant difference, but the statistical analysis indicates that this difference can be easily accounted for by inherent sampling variability.

SUMMARY AND CONCLUSIONS

(a) Ninety-six dogs were exposed to an LD_{85} of total body x-irradiation. Those treated by the prophylactic administration of Aureomycin (50 mg. per Kilogram of body weight per day) and/or the transfusion on a demand basis of stored whole blood were neither benefited nor affected adversely. (b) Ninety-six dogs were exposed to an LD_{62} of total body x-irradiation. Those treated by the prophylactic administration of Aureomycin (75 mg. per Kilogram of body weight per day) and/or the transfusion on a predetermined basis of stored whole blood were not affected by the therapy. (c) Possible reasons for the failure of the therapy are discussed. (d) Since, in contrast to the animals used in this study, the human casualty of an atomic catastrophe would more likely be exposed to infection, the prophylactic administration of a broad spectrum antibiotic is still advisable despite the results of this study. (e) Since transfusion of stored whole blood did not prevent or limit the extent of the hemorrhagic diathesis, such therapy should not be used prophylactically. It can only be recommended for the replacement of the blood volume or for the treatment of a severe anemia caused by hemorrhage.

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THE EFFECT OF GASTRIC ACIDITY ON THE ABSORPTION OF QUINIDINE SULFATE FROM THE GASTROINTESTINAL TRACT

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EXPERIMENTAL studies[1, 2] have shown that plasma concentrations of quinidine may vary markedly in different individuals following the same oral dose of quinidine sulfate. Quinidine is an isomer of quinine, and the two drugs have many chemical properties in common. In the tropics, dilute acid is often given along with quinine during treatment of malaria. Since the urinary excretion of quinine has been noted to be increased following the administration of acid, it was thought that there might be some relation between gastric acidity and the absorption of quinine and its isomer, quinidine.[3] The question arose as to whether gastric acidity plays any part in the absorption of quinidine. In an effort to find the answer, quinidine concentrations in the plasma of people with normal gastric acidity and in patients with achlorhydria were determined following the administration of a single oral dose of quinidine sulfate.

MATERIALS AND METHODS

The normal individuals (Group 1) consisted of 20 healthy medical students whose ages ranged from 21 to 31 years, the average being 23 years. Gastric acidity was determined in the fasting state and again following the administration of 250 ml. of 7 per cent ethyl alcohol. Each of the normal persons had free hydrochloric acid in both specimens. The mean values for free hydrochloric acid in the group were 10.2 clinical units in the fasting specimen and 38.1 clinical units following alcohol.

Group 2 was made up of 16 convalescent patients with achlorhydria from the general medical wards of the North Carolina Baptist Hospital, the Forsyth County Hospital, and the Forsyth County Home. Their ages ranged from 36 to 95 years, averaging 69.7 years. None had free hydrochloric acid in the fasting state or after the subcutaneous administration of 0.1 mg. of histamine base.

Quinidine absorption curves on both groups were done with the subjects fasting. Determinations of the plasma quinidine concentrations, utilizing the method of Brodie and Udenfriend,[4] were made before, one-half, one, two, three, and four hours after the oral administration of 0.4 Gm. of quinidine sulfate in a gelatin capsule.

In order to eliminate the error which might result from the presence of other substances, such as vitamins, which appear fluorescent under ultraviolet light, the pretreatment sample was used as a control in each case, the photofluorometer (Coleman) being adjusted to register 0 on this sample before the later plasma samples were read.

RESULTS

The results of the study are shown in Table I. The plasma concentrations of quinidine were consistently higher in the group with achlorhydria than in the control group, the greatest difference in the means being approximately 2.0

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mg. per liter at the second and third hours. This difference is not statistically significant ($P > 0.01$).

COMMENT

It would seem that quinidine is easily absorbed, regardless of the degree of gastric acidity. Since both free quinidine and quinidine sulfate are soluble in small amounts of water over a wide range of pH,[5] precipitation in the gastrointestinal tract seems unlikely as an explanation for the wide range in plasma levels which has been observed. Taggart and his co-workers[2] reported that less than 5 per cent of serially administered doses could be recovered from the stool. A variation in the rate of utilization and excretion of quinidine from one individual to another could explain the discrepant plasma levels, but this factor was not investigated in this study.

SUMMARY

To determine the effect of gastric acidity on the absorption of quinidine sulfate, absorption curves following the administration of a single oral dose of quinidine sulfate (0.4 Gm.) were determined during the fasting state on 20 healthy medical students with normal acidity and 16 patients with achlorhydria. While the quinidine concentrations were consistently higher in the group with achlorhydria, the difference between the mean values for each group is not statistically significant ($P > 0.01$), and it is felt that the degree of gastric acidity is not a factor in the absorption of quinidine sulfate from the gastrointestinal tract.

TABLE I. PLASMA QUINIDINE CONCENTRATIONS IN SUBJECTS WITH NORMAL GASTRIC ACIDITY AND PATIENTS WITH ACHLORHYDRIA, FOLLOWING THE ORAL ADMINISTRATION OF 0.4 GM. OF QUINIDINE SULFATE

TIME AFTER ADMINISTRATION (HR.)	GROUP	NO. SUBJECTS	RANGE (MG. PER L.)	MEAN (MG. PER L.)	STANDARD DEVIATION
½	1. Normal	20	0-1.90	0.65	0.620
	2. Achlor.	16	0.23-4.04	1.64	0.955
1	1. Normal	20	0.23-2.85	1.54	0.740
	2. Achlor.	16	1.42-5.94	3.26	1.148
2	1. Normal	20	0.95-3.57	2.09	0.654
	2. Achlor.	16	1.90-6.18	4.12	1.147
3	1. Normal	20	1.42-3.09	2.05	0.460
	2. Achlor.	16	1.90-6.66	3.98	0.832
4	1. Normal	20	1.19-2.85	1.95	0.431
	2. Achlor.	16	1.66-6.78	3.47	1.107

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THE NEUTRALIZATION OF AMINOPTERIN-INDUCED LEUKOPENIA BY ADENOSINE-5-PHOSPHORIC ACID

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THE depressant effect upon the number of circulating leukocytes of compounds such as aminopterin, urethane, mechlorethamine hydrochloride, and triethylene melanine is of value when these compounds are tried therapeutically against chronic leukemia, but is a distinct disadvantage when these compounds are tried in other conditions such as malignancy and acute leukemia[13] where the numbers of circulating leukocytes are normal or below normal. Should a compound be encountered in these or related chemical groups which is useful in the treatment of malignancy, it seems quite likely that it would exhibit a leukopenic action as an undesired side effect. It is useful, therefore, to have available drugs which neutralize these leukopenic side reactions. In the investigation to be reported below, it was found that simultaneous administration of adenosine-5-phosphoric acid prevented the development of leukopenia in guinea pigs induced by injection of aminopterin. The use of adenosine-5-phosphoric acid did not lower the mortality rate.

METHOD

Leukopenia was produced in guinea pigs by subcutaneous injection of aminopterin after the technique of Innes, Innes, and Moore,[6] except that larger doses were administered for a shorter period of time. The experiments were performed upon large, male guinea pigs, of one pound or more body weight, fed upon Purina rabbit chow supplemented with greens. Before drug treatment, the red blood cells and white blood cells were counted by means of a Levy chamber,[15] hemoglobin determined by the direct photometer method,[4] and a differential white blood cell count made with Wright's stain. The normal values, listed in Table I, approximate those of King and Lucas[11] with respect to the differential leukocyte count, while the remaining values correspond to those reported by Innes, Innes, and Moore.[6]

A sterilized 1.0 per cent solution of aminopterin in 0.1N sodium bicarbonate was prepared for each experiment and injected subcutaneously, in doses of 5 and 10 mg. of aminopterin per kilogram of body weight per twenty-four hours for ten days, into two groups of 10 and 15 guinea pigs each, respectively. Blood counts and hemoglobin estimations were made at intervals of two to five days during and for ten days after treatment with aminopterin. Forty per cent of the animals receiving 5 mg. per kilogram and 67 per cent of those receiving 10 mg. per kilogram of aminopterin died during or shortly after treatment, following considerable loss in weight and cachexia. Both doses of aminopterin produced statistically significant ($P<0.01$) decreases in the number of white blood cells, as calculated after Bradford Hill.[5] Typical results at the end of ten days of treatment are shown in Table I. There were decreases in the mean numbers of red blood cells and in the concentration of hemoglobin which were not sufficiently marked to be statistically significant. The leukopenia affected all types of white blood cells with a statistically significant greater loss of neutrophils than of lymphocytes. The reaction was similar to that reported by Innes, Innes, and Moore.[6]

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Adenosine-5-phosphoric acid was dissolved in distilled water, with the addition of 0.1N sodium hydroxide to pH 4 to effect solution when required, sterilized by heating to 100° C. for fifteen minutes, and injected subcutaneously daily for ten days into four groups of ten guinea pigs each, in doses of 0.1, 5.0, 25.0, and 100 mg. per kilogram of body weight, respectively. The hemoglobin content, numbers of red blood cells and white blood cells, and the differential leukocyte count were determined before, and at intervals of two to five days during and after the ten-day period of treatment. The data were tabulated, means and standard deviations calculated, and representative values at the end of ten days of treatment have been listed in Table I.

TABLE I. THE EFFECT OF SUBCUTANEOUS INJECTION DAILY FOR TEN DAYS OF AMINOPTERIN AND/OR ADENOSINE-5-PHOSPHORIC ACID UPON THE RED BLOOD CELLS, HEMOGLOBIN, WHITE BLOOD CELLS, AND DIFFERENTIAL WHITE BLOOD CELL COUNT OF GUINEA PIGS

DOSE (MG. PER KG.)	NO. OF ANIMALS	CALCULATION	RED BLOOD CELLS ($\times 10^6$ PER CU. MM.)	HEMOGLOBIN (GM. PER 100 ML.)	WHITE BLOOD CELLS (PER CU. MM.)	DIFFERENTIAL COUNT—PER CENT				
						NEUTROPHILS	EOSINOPHILS	BASOPHILS	MONOCYTES	LYMPHOCYTES
Untreated										
		Mean	5.77	12.74	11,570	26.8	2.4	0.9	4.5	65.4
0.0	169	Standard Deviation	0.97	1.87	2,950	3.4	1.7	1.1	3.6	3.5
Aminopterin—Treated										
5.0	10	Mean	4.70	9.63	3,230	21.7	1.9	0.6	6.4	69.4
10.0	15	Mean	4.92	12.68	2,330	13.7	1.4	0.8	2.6	81.5
Adenosine-5-Phosphoric Acid—Treated										
0.1	10	Mean	4.91	14.03	10,900	25.0	1.4	1.6	9.7	62.3
5.0	10	Mean	5.57	10.05	11,400	31.7	0.7	0.5	5.3	61.9
25.0	10	Mean	6.24	12.82	11,100	36.9	3.5	0.7	5.2	53.7
100.0	10	Mean	5.65	11.26	17,800	38.5	1.2	0.3	7.1	52.7
Aminopterin (5 mg. per kg.) + *Adenosine-5-Phosphoric Acid* (100 mg. per kg.)										
—	20	Mean	3.27	5.77	8,080	31.5	2.2	1.3	6.0	59.0

RESULTS

Administration of adenosine-5-phosphoric acid had no significant effect upon the numbers of red blood cells nor upon the blood hemoglobin content. Doses of adenosine-5-phosphoric acid of 0.1, 5.0, and 25.0 mg. per kilogram of body weight also had no significant effect upon the number of white blood cells. The dose of 100 mg. per kilogram killed 30 per cent of guinea pigs, produced a leukocytosis, affecting chiefly the neutrophils, and the mean increase in the number of white blood cells had a P value less than 0.01.

Adenosine-5-phosphoric acid, or muscle adenylic acid, is known to be involved as a phosphate donor-acceptor in many cellular reactions and in doses five to ten times greater than the maximal dose used above will produce hemoconcentration, shock, and death in mice, rats, and rabbits.[3] That hemoconcen-

tration could not account for the leukocytosis noted above is manifested in the lack of increase in the number of red blood cells and hemoglobin. Neither did the animals appear to be in a shock sta e, but there was noted a decrease in bleeding volume after several days of treatment.

That the leukocytosis induced by injection of adenosine-5-phosphoric acid was probably not directly related to blood levels of the administered compound is suggested by the work of Albaum, Cayle, and Shapiro.[1] These authors found that human blood nucleotide adenine, averaging some 7 mg. per 100 ml., could be increased by intramuscular injection of 20 mg. of adenosine-5-phosphoric acid, but that the augmentation was not appreciably increased by quintupling the administered dose. On the other hand, they found that administration of yeast adenosine-3-phosphoric acid did not augment blood levels of nucleotide adenine, which may have a bearing upon the equivocal value of pentnucleotide from yeast in the treatment of agranulocytosis. These and reports of the growth stimulating action of adenine nucleotides[7] and their stimulation of blood formation[12] were among the observations which prompted trial of adenosine-5-phosphoric acid in the study herein reported.

Combination treatment of guinea pigs with aminopterin and adenosine-5-phosphoric acid was tried with various doses of each compound. The combination found to be most effective in preventing aminopterin-induced leukopenia was subcutaneous injection daily of 5 mg. per kilogram of body weight of aminopterin and 100 mg. per kilogram of body weight of adenosine-5-phosphoric acid for ten consecutive days. Results of treatment of 20 guinea pigs with this combination are given in Table I.

As may be seen, the administration of this dose of adenosine-5-phosphoric acid prevented the development of a leukopenia in the guinea pigs. These results may be said to correspond with the observation of Naber, Snell, and Cravens[8] that certain derivatives of nucleic acid are capable of reversing aminopterin inhibition of embryonic development in the chick. At the same time, however, there was a fall which averaged 45 per cent in the number of red blood cells and which averaged 55 per cent in the hemoglobin concentration. By the tenth day, 45 per cent of the animals had died. All treatment was then stopped, and five days later only 3 of the 20 guinea pigs were still alive.

The experiment was repeated upon a further 10 guinea pigs, given daily for ten days by mouth, 200 mg. per kilogram of body weight of iron and ammonium citrate, as 2 ml. per kilogram of a 10 per cent solution in distilled water, in addition to the subcutaneous injections of 5 mg. per kilogram of aminopterin and 100 mg. per kilogram of adenosine-5-phosphoric acid. The administration of this dose of iron prevented development of the secondary anemia described above. The administration of the adenosine-5-phosphoric acid again prevented the appearance of a leukopenia. In none of these animals was there any significant alteration in the blood values determined. Nevertheless the guinea pigs still died at the same rates and times as noted in the previous paragraph. Autopsy of these animals revealed no gross abnormalities in the thorax or abdomen; microscopic sections were not made.

The experiment described in the previous paragraph was repeated upon 10 guinea pigs which received daily for ten days an intramuscular injection of 20,000 units of penicillin G potassium, in addition to the iron, aminopterin and adenosine-5-phosphoric acid. The concentration of hemoglobin, the number of red blood cells and the number and types of white blood cells all remained within pre-treatment limits. The death rate, however, was not lowered and at autopsy no cause of death was found.

DISCUSSION

The cause of death following injection of antifolic acid compounds is generally believed to be a progressively developing panleukopenia with consequent lack of resistance, manifested in ulcerative ileitis and colitis and secondary infections.[2] As demonstrated above, simultaneous administration of adenosine-5-phosphoric acid in sufficient dose will prevent development of leukopenia but will not prevent death, which is due, at least in part, to the large dose of adenosine-5-phosphoric acid required. Adrenalectomy minimizes the toxic effects of aminopterin in rats.[10]

Administration of the citrovorum factor has been reported to prevent toxic reactions to aminopterin in mice and rats[9] and to prevent development of aminopterin-induced leukopenia.[14] These results, which have been confirmed by several investigators, obviate the urgency of finding another antidote to the leukopenic action of aminopterin but it would be useful to have a substitute corrective in case the original failed to function.

SUMMARY

Simultaneous administration of adenosine-5-phosphoric acid was found to prevent aminopterin-induced leukopenia in guinea pigs but not to prevent death. Subcutaneous injections of aminopterin daily for ten days, in doses of 5 and 10 mg. per kilogram of body weight, produced a fall in the number of circulating blood leukocytes in 25 guinea pigs to levels which were significantly lower than those of 169 untreated controls, the reaction affecting chiefly the neutrophils and being accompanied by no significant anemia and a mortality of some 50 per cent. Injection of adenosine-5-phosphoric acid alone into 40 guinea pigs in doses of 0.1 to 25.0 mg. per kilogram had little or no effect, but a dose of 100 mg. per kilogram produced a leukocytosis affecting chiefly the neutrophils, no anemia, and 30 per cent mortality. Simultaneous administration of 5 mg. per kilogram of aminopterin and 100 mg. per kilogram of adenosine-5-phosphoric acid to 40 guinea pigs resulted in no change in the circulating leukocytes, a marked secondary anemia and a mortality of 45 per cent. Simultaneous administration of iron, with or without penicillin, prevented development of the anemia but did not lower the mortality.

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A METHOD OF QUANTITATIVE DIFFERENTIAL AGGLUTINATION OF DOG ERYTHROCYTES

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THE purposes of this paper are to describe and to analyze the accuracy of a method of quantitative differential agglutination of dog erythrocytes.

Differential agglutination of erythrocytes has been applied extensively to studies of hemolytic mechanisms and blood preservation. There are serious limitations, however, to the widespread use of this method in human subjects, especially when the transfusions involve potential hazard to the volunteer recipient. It therefore seemed of value to adapt this technique to the dog, in which animal there are precisely characterized erythrocyte antigens and isoantibodies.

Canine erythrocyte isoantibodies of 5 specificities have been characterized in detail, and designated anti-A, -B, -C, -D, and -E in the order of their demonstration in this laboratory.[1] Two additional isoantibodies have been demonstrated and several other immune isoantibodies are under investigation.[2] Canine anti-A is a potent hemolysin in vivo and in vitro and is capable of sensitizing A_1 erythrocytes for the antiglobulin test. Most examples of anti-B, -C, and -D act only as agglutinins. Anti-E readily sensitizes erythrocytes for the antiglobulin reaction, and is a potent agglutinin as well. These antibodies have been produced by immunization of dogs with erythrocytes containing antigenic factors which the recipient animal lacks. Small amounts of "naturally occurring" isoantibody, most fequently anti-D, have been found in dogs that have not been artificially immunized.[3]

It is of interest that the first attempt to differentiate quantitatively autogenous and transfused erythrocytes by serological means was carried out in cattle by Todd and White in 1910. These workers used a hemolytic isoantibody produced by immunization of cattle incident to the production of a vaccine.[4, 5] Ashby, using human isoantibodies, in 1919, first applied this technique to the study of human red cell survival.[6] The technique has been modified subsequently by a number of workers using a variety of antibodies.[7-11] The statistical and mathematical aspects of the method have been discussed in detail in several recent publications.[12-18] Results of studies employing differential agglutination of dog erythrocytes have been reported previously from this laboratory,[19] and by Hamilton and Ravdin,[20] and by Brown,[21] but details of the method as applied to this species have not heretofore been described.

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Wright and co-workers have recently reported observations on quantitative differential agglutination of rabbit erythrocytes.[22]

MATERIALS AND METHODS

Several methods of differential agglutination were employed before the following technique, shown diagrammatically in Fig. 1, was adopted. A dry, 3 inch, long-bevel 20-gauge needle is inserted into the animal's external jugular vein, using minimal venous stasis. After release of venous stasis, 3 ml. of blood are withdrawn into the attached dry syringe. The syringe is then removed from the needle, leaving the needle in place in the vein. This sample of blood is added to a mixture of the dry salts, 3.6 mg. of ammonium oxalate and 2.4 mg. of potassium oxalate. The hematocrit is determined from this sample by the Wintrobe method,

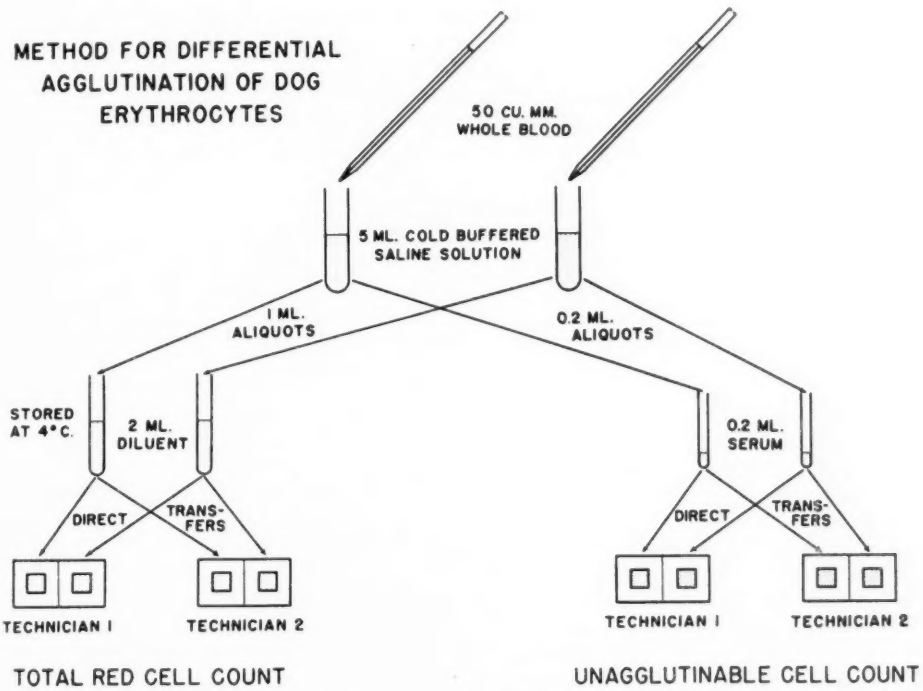

Fig. 1.—Diagram of the steps in the technique of differential agglutination of dog erythrocytes.

and the remainder is used for other hematologic determinations. Two 50 cubic millimeter pipettes* are filled directly from the continuing flow of blood from the needle hub. Each of these pipettes is emptied into a separate 13 by 100 mm. tube containing 5 ml. of cold, buffered† isotonic saline solution and thoroughly mixed. Since no difficulty with fibrin clot formation is encountered with this dilution of whole dog blood, no anticoagulant is used.

These cell suspensions in buffered saline are immediately mixed by inversion and rotation for one minute. A 1 ml. aliquot is then removed from each tube and added to separate 13 by 100 mm. tubes containing 2 ml. of a formaldehyde-citrate solution (1 per cent

*Fifty cubic millimeter pipettes with correction factors for content volume were furnished by the W. H. Stewart Laboratory Supply Co., Rochester, N. Y. Erythrocyte counts were corrected for these errors of calibration. An effort was made to use pipettes requiring no such correction.

†Buffered isotonic saline solution was prepared from the following buffer mixture:

7 parts M/11 Na_2HPO_4
3 parts M/11 NaH_2PO_4

1 part of this buffer mixture was added to 9 parts of 0.85 per cent NaCl solution. The final pH of this solution is 7.0.

formaldehyde and 3 per cent sodium citrate). These latter tubes are then stored at 4° C. until used to determine the total red cell count. Another aliquot of 0.2 ml. is removed from each of the cell suspensions in buffered saline and placed in separate 7 by 75 mm. tubes, to each one of which 0.2 ml. of agglutinating serum is then added. All measurements are made with volumetric pipettes.

The serum-cell mixtures are allowed to stand for fifteen minutes at room temperature. They are then centrifuged at 1,000 r.p.m. for one minute after which the tubes are vigorously agitated by hand and recentrifuged. The packed cell mass is broken up by shaking the tube lightly ten times. A few seconds are allowed for the larger clumps of agglutinated cells to settle; then a sample is removed from the tube using a large-bore capillary tube (1.2 mm. internal diameter) allowed to fill by capillary action. This sample is used to fill one counting chamber. The same tube is again shaken lightly five times and a second specimen is similarly removed with a clean capillary tube and placed on another counting chamber. The cells from the second agglutination tube are likewise placed on the other halves of these two counting chambers. The capillary tubes are discarded after being used once.

Counting is carried out by two technicians who enumerate the unagglutinated cells in a minimum of ten red cell counting squares, the dimensions of each square being 0.2 by 0.2 mm., with a depth of 0.1 mm. An attempt is made to have each technician count the red cells in a volume large enough to contain at least 500 cells on each side of the chamber. The average count is expressed as the number of cells per 5 squares.

Total red cell counts are determined from the two previously prepared tubes containing the 1:3 dilution with formaldehyde-citrate solution of the original cell suspensions. Each of these tubes is mixed by rotation and inversion for one minute. Samples are removed from each tube with capillary tubes, transferred to counting chambers, and enumerated in the same fashion as the unagglutinable cells are counted. A minimum of 500 cells (in at least 5 squares) are counted on each side of a chamber by each technician.

Calculations.—

(1) *Total red cell count:*

$$\text{Average number of cells per 5 squares in 1:3 dilution of original suspension} \times 15{,}000 = \text{total number of red cells per cu. mm. of whole blood}$$

(2) *Unagglutinable or "donor" cell count:*

$$\text{Average number of unagglutinated cells per 5 squares from agglutination tubes} \times 10{,}000 = \text{number of unagglutinable cells per cu. mm. of whole blood}$$

(3) $$\text{Per cent unagglutinable or "donor" cells} = \frac{\text{unagglutinable cell count/cu. mm. whole blood}}{\text{total red cell count/cu. mm. whole blood}} \times 100$$

(4) $$\text{Per cent agglutinable or "recipient's" cells} = 100 - \text{per cent unagglutinable or "donor" cells}$$

(5) $$\text{"Donor" cells/cu. mm. of red cells} = \text{unagglutinable cells/cu. mm. whole blood} \times \frac{100}{\%\ \text{hematocrit}}$$

Unagglutinable cell counts may be expressed as number per cubic millimeter of whole blood, number per cubic millimeter of red cells, or as per cent of total red cells, according to the requirements of a particular experiment. If only the number of donated red cells per cubic millimeter of red cells is desired, the total red cell count may be eliminated. The number of counts necessary for each determination is thus reduced and technician fatigue is minimized. This method of expression of unagglutinable cell counts has the further advantage of minimizing variability of results introduced by changes in plasma volume of the recipient.

TABLE I. INCIDENCE OF REACTIONS OF RED CELLS OF RANDOM MONGREL DOGS WITH CANINE ERYTHROCYTE ANTISERA

ANTISERUM	NUMBER DOGS TESTED	NUMBER DOGS POSITIVE	PER CENT POSITIVE
Anti-A	714	438	61
Anti-B	714	38	5
Anti-C	714	703	98
Anti-D	575	134	23
Anti-E	334	225	76

Production and Testing of Dog Isoantibodies.—Antisera of four specificities, anti-B, -C, -D, and -E, were used in these studies. Table I gives the incidence in the erythrocytes of random mongrel dogs of the antigens detected by these antibodies. Anti-B which reacts with a small proportion, and anti-C which reacts with nearly all random dogs' erythrocytes are of less value in transfusion studies because of difficulty in finding suitable donors and recipients. The first serum which was found to be satisfactory for quantitative differential agglutination was of anti-D specificity, and since the distribution of this factor in random dogs' erythrocytes is favorable, this report principally concerns our observations using this antibody. Only one example of anti-E of sufficient potency has been available, and our experience with this serum is limited. High unagglutinable cell counts have been obtained with all canine anti-A sera thus far studied, regardless of titer. Quantitative differential hemolysis by the potent lytic action of canine anti-A in the presence of complement also has been unsuccessful. Fractionation and concentration by lyophilization have not improved the performance of any of these isoantibodies. Attempts to produce satisfactory antibodies in rabbits for differential agglutination of dog erythrocytes have thus far failed.

All antisera were produced by intravenous injection of 3 to 5 ml. quantities of whole blood containing the desired antigens. Injections were given weekly in repeated courses of 5 to 8 doses. Titrations of antibody content were made by the method previously described.[19] Serum was collected in quantity when found to be satisfactory for differential agglutination. All sera were heated at 56° C. for thirty minutes, and sodium azide added to a final concentration of 1:1000 before being frozen and stored at -20° C., in 3 to 4 ml. portions.[23] Sera with titers of 1:128 to 1:512 were obtained, and remained fully potent on prolonged storage in the frozen state.

Sera were tested by carrying out unagglutinable cell counts by the same technique used for differential agglutination. A 1:100 dilution of whole blood from a single dog was employed in such tests. All sera were required to leave unagglutinated less than 30,000 cells/cu. mm. of whole blood from this dog. From 5,000 to 40,000 D+ canine red cells per cubic millimeter of whole blood from D+ dogs selected at random were found to be unagglutinable with anti-D serum of adequate potency; the average unagglutinable cell count was about 15,000 cells per cubic millimeter of whole blood. These values are comparable to those obtained when potent human anti-A sera are used to agglutinate human A_1 erythrocytes.

Only a small proportion of -B, -D, or -E negative dogs will produce antiserum satisfactory for differential agglutinations of red cells. In one group of 10 D dogs given D+ erythrocytes, only two animals produced anti-D antibodies, and only one of these was sufficiently potent for quantitative differential agglutination. The effect of a variety of means of introducing the antigenic cells on the production of antibodies is under investigation. At the present time, it would appear that the difficulty of producing adequate sera constitutes the principal limitation to the widespread use of this technique.

Studies have been conducted to delineate optimal conditions for maximal agglutination of red cells by anti-D serum. Fifteen minutes of exposure of cells to serum at room temperature seems optimal, although not critical. At 37° C. some artificial hemolysis may occur. Prozones have not been detected with these sera. Dilutions of 1:2 or 1:4 can be made with the most potent sera as a measure to conserve serum supplies. Vigorous agitation of agglutinated cells rapidly reduces the size of the agglutinated masses, but does not increase the true unagglutinable cell count.

It has been the experience of several investigators in this and other laboratories that dog erythrocytes, in contrast to human red cells, hemolyze relatively easily when used in a variety of laboratory procedures. Dog erythrocytes are especially sensitive to minute traces of soaps, detergents, and cleaning solutions, and these must be scrupulously avoided in quantitative studies. Clean, dry needles, syringes, and Pyrex glassware are employed, and cleaning is carried out by copious rinsing with water only immediately after equipment is used. Anticoagulants are avoided in so far as possible because their effects upon canine erythrocytes are not well known. Suspensions of dog erythrocytes in the buffered isotonic saline solution utilized in these studies have been stable for periods up to twelve hours when kept at 4° C. In practice, these suspensions are utilized as soon as possible, usually within less than one-half hour, and no difficulties with artificial hemolysis have been observed.

The technique of differential agglutination of human erythrocytes described by Young, Platzer, and Rafferty[11] has been applied to dog erythrocytes and compared with the method presented here in a few instances. Comparable results were obtained by both methods in these limited observations. Nevertheless, we feel that the modifications introduced in adapting the method to the dog are useful safeguards, especially in situations where artificial hemolysis may occur because of unavoidable delay in counting specimens.

The number of recipient's unagglutinable cells has been determined prior to transfusion with the same lot of serum to be used during the subsequent transfusion experiment. Unagglutinable cell counts post transfusion are corrected by subtracting this initial "blank" count. This correction is significant only when small numbers of cells are transfused or survive. Although it has not been shown directly in the case of the dog that such a correction remains constant after introduction of foreign unagglutinable cells into the agglutination reaction, it seems probable that this is the case. A correction is therefore made, as recommended by most workers engaged in studies on differential aggultination of human red cells.

RESULTS

Studies on mixtures of agglutinable and nonagglutinable dog erythrocytes.—Studies have been carried out with mixtures of agglutinable and nonagglutinable cells in order to evaluate the accuracy of the differential agglutination step of this procedure. These mixtures were prepared from dilute suspensions of the two types of cells made by adding fresh whole dog blood directly to cold buffered isotonic saline solution. Red cell counts of these suspensions were made by utilizing especially calibrated white cell counting pipettes and the photographic red cell counting method described in an accompanying paper.[24] Dilutions and mixtures of these erythrocyte suspensions were made rapidly using volumetric glassware or accurately calibrated serologic pipettes. Mixtures of red cells prepared in this fashion are likely to be more accurate in composition than those made directly from whole blood because of the much greater difficulty in accurate counting of the red cells in whole blood. Large errors in composition of the mixture may be introduced, moreover, by slight errors of pipetting whole blood. Burettes should not be used for such volumetric measurements as sedimentation of the cells will result in gross inhomogeneity of the suspension being measured. Photographic counting was utilized in these experiments because of the large number of red cell counts to be made. In similar experiments, where smaller numbers of counts were made, the direct visual method of counting was employed using larger numbers of technicians. Results obtained with both methods of erythrocyte counting have been comparable.

TABLE II. DIFFERENTIAL AGGLUTINATION STUDIES OF ARTIFICIALLY PREPARED MIXTURES OF AGGLUTINABLE AND UNAGGLUTINABLE CANINE

TABLE II. DIFFERENTIAL AGGLUTINATION STUDIES OF ARTIFICIALLY PREPARED MIXTURES OF AGGLUTINABLE AND UNAGGLUTINABLE CANINE ERYTHROCYTES

EXPERIMENT NUMBER	DESCRIPTION OF EXPERIMENT	COUNT OF RED CELLS IN SUSPENSION BEFORE MIXING		APPROXIMATE PER CENT CELL TYPE IN MIXTURE		PREDICTED UNAGGLUTINABLE CELL COUNT OF MIXTURE PER CU. MM.	OBSERVED UNAGGLUTINABLE CELL COUNT OF MIXTURE PER CU. MM.	NUMBER OF DETERMINATIONS OF UNAGGLUTINABLE CELL COUNT	DEVIATION OF OBSERVED VALUE FROM PREDICTED VALUE
		AGGLUTINABLE CELLS PER CU. MM.	UNAGGLUTINABLE CELLS PER CU. MM.	AGGLUTINABLE CELLS	UNAGGLUTINABLE CELLS				
1	Mixture of approximately 50% D-positive and 50% D-negative cells tested with Anti-D serum	(approx.) 44,500	(Mean of 18 Counts) 42,800 ± 770*	50	50	21,400 ± 385	22,070 ± 203	31	+670 (+3.1%) ±435†
2	Mixtures containing approximately from 5% to 40% D-negative cells tested with Anti-D serum	(approx.) 41,400	(Mean of 20 Counts) 36,600 ± 810	60	40	14,650 ± 324	14,500 ± 422	20	-150 (-1.0%) ±623
				70	30	10,980 ± 243	9,860 ± 134	20	-1,120 (-10.1%) ±278
				80	20	7,320 ± 162	6,670 ± 108	10	-650 (-8.9%) ±195
				90	10	3,660 ± 81	3,420 ± 104	10	-240 (-6.6%) ±132
				95	5	1,830 ± 41	2,050 ± 114	10	+220 (+12.0%) ±121
3	Comparison of Anti-B, Anti-D, and Anti-E on a mixture containing approximately 25% of cells unagglutinable by any of the three sera	(approx.) 39,200	(Mean of 28 Counts) 40,100 ± 627	75	25	10,250 ± 157	With Anti-B 9,840 ± 161	10	-410 (-4.0%) ±224
							With Anti-D 10,610 ± 266	10	+360 (+3.5%) ±309
							With Anti-E 9 770 ± 123	10	-480 (-4.7%) ±200

*Standard error of mean
†Standard error of difference.

Three representative experiments are outlined briefly in Table II along with the results obtained. It will be seen that the differences between observed and predicted values cover a range of approximately ±10 per cent. These differences do not take into account possible errors of sampling or pipetting which might have affected the accuracy of the predicted values, since the mixtures were prepared only once for each experiment. In two instances in Experiment 2, the deviation of the observed value from the predicted value is substantially greater than two times the standard error (S.E.) of the difference. This failure of agreement may well be due to errors of volumetric measurement or sampling encountered in preparing these mixtures of the two cell suspensions. The fact that excellent agreement of observed and predicted values was obtained with mixtures containing both more and fewer unagglutinable cells supports the view that the error lies in the predicted values in these cases. In all other instances, the differences noted are no greater than can be accounted for by the inherent variability of the experiment, which is largely that of red cell counting and sampling.

Mollison has recently described an experiment utilizing artificially prepared mixtures of agglutinable and unagglutinable erythrocytes in which errors of volume measurement are minimized by use of the same pipette for all duplicate measurements.[25] Table III gives the results of such an experiment carried out with another canine anti-D serum. It will be seen that again there is no significant difference between observed and predicted values for the number of agglutinable cells. Approximately 20 per cent of unagglutinable cells were present in the mixed cell suspension used in this experiment, as in one of the experiments recorded in Table II. The good agreement of observed and predicted values of unagglutinable cell count in this experiment suggests further that the small differences encountered in the former experiment are not errors introduced by the differential agglutination. It would seem justifiable to conclude that the differential agglutination step of this technique is quantitative and specific with the canine antisera tested.

The other sources of error encountered in differential agglutination studies, in addition to those introduced by the differential agglutination step itself, are well recognized. These are the errors of red cell counting, and errors of sampling and volumetric measurement. Errors of this type can be minimized by

TABLE III. ARTIFICIALLY PREPARED MIXTURE OF APPROXIMATELY 20% D+ AND 80% D- CANINE ERYTHROCYTES DIFFERENTIALLY AGGLUTINATED WITH CANINE ANTI-D SERUM. (EXPERIMENT PERFORMED BY METHOD OF MOLLISON[25])

	Cells per 5 counting squares*
Predicted unagglutinable cell count of mixture	301 ± 4.2† (6028 cells counted)
Observed unagglutinable cell count of mixture	298 ± 4.4† (5969 cells counted)
"Blank" unagglutinable cell count of D+ red cell suspension	3 (648 cells counted)
Difference (Predicted + "Blank" - Observed)	6 ± 6.0‡

*Dimensions of the squares are 0.2 by 0.2 by 0.1 mm.
†Standard error of mean.
‡Standard error of difference.

statistical methods and careful attention to technique. In contrast, the error introduced by differential agglutination depends basically upon the characteristics of the serum employed.

DISCUSSION

The post-transfusion survival of normal and stored canine erythrocytes determined by the technique described here is reported in an accompanying paper.[26] We have utilized this method in our laboratory in over 40 transfusion experiments in dogs and have found the method reliable and simple. Dogs are easily bled, transfused, and cared for, and are generally available. The rate of elimination of several donor cell populations can be measured simultaneously in one recipient, as in man, by use of agglutinating sera of different specificities. This type of experiment requires access to large numbers of dogs so that compatible donors and recipients of appropriate blood type can be chosen.

This method has been used in our laboratory to study hazardous transfusion experiments such as those involving administration of serologically incompatible blood. Other procedures, such as splenectomy, construction of renal-bile fistulas and administration of large amounts of radioactive tracers can be carried out in connection with studies employing the Ashby technique in dogs. A study of the mechanism of the development of anemia following lethal whole body x-irradiation of dogs has been conducted, utilizing this technique.[27]

Two major difficulties have been encountered in the course of these studies, chief of which has been that of producing satisfactory antisera. This problem may be overcome by immunizing many dogs and collecting serum in large quantities from the dogs exhibiting most satisfactory antibody production.

The second major difficulty encountered has been the development of isoantibodies in the recipient in response to transfusion of red cells from the donor dog, thus making the donor cells incompatible and possibly subject to accelerated destruction. In an effort to avoid this complication, prospective donors are rejected if their red cells contain any of the 7 known antigenic factors that are found to be lacking in the red cells of the prospective recipient dog. Particular care must be taken to avoid transfusing A– dogs with A+ cells since the A factor is highly antigenic and anti-A is a potent hemolysin. In spite of care in selecting donor-recipient combinations and in cross-matching prior to transfusion, previously unidentified antigenic factors in the donor cells may occasionally immunize the recipient and cause rapid destruction of the donated erythrocytes. This complication may be much like that occasionally encountered with minor blood group differences in human transfusions.

After all donated erythrocytes have been eliminated from the recipient's circulation, the recipient's plasma should be tested again with the donor's erythrocytes, as in the pretransfusion cross-match, to determine whether or not the recipient dog has developed an isoantibody as a result of the transfusion. In certain types of experiments with "treated" donor cells, the recipient dog should be transfused with untreated erythrocytes from the same donor after the treated cells have disappeared from the recipient's circulation. The transfusion with untreated cells, the destruction of which is measured by differential

agglutination, serves as a control by demonstrating in vivo the presence or absence of significant incompatibility between donor and recipient. The need for such controls was illustrated in several transfusion experiments in this laboratory in which there was significantly shortened survival of the donated red cells despite negative in vitro matching tests, including the usual tests for incomplete antibodies. It is possible that such results may be due to intrinsic abnormalities of the donor erythrocytes, or to the presence of an abnormal lytic mechanism in the recipient unrelated to isosensitization.

In some instances an isoantibody developed as a result of transfusion may *not* shorten the survival period of the donated red cells. For example, C+ dog erythrocytes were found to survive normally in the circulation of one immunized C– recipient dog, despite the fact that the recipient's serum was capable of agglutinating the donor's red cells at body temperature in vitro.[28]

An additional precaution that should be taken in studies on survival of transfused dog erythrocytes is that of selecting a donor whose red cell population can be expected to have an essentially normal age distribution. A donor dog that has been bled recently or otherwise rendered anemic should therefore not be used. The life span of red cells produced during periods of stress on the erythroid elements of the bone marrow is not well defined in the dog or in other species.

Each of the precautions taken in connection with studies on differential agglutination of dog erythrocytes must also be taken in conducting comparable studies in other species including man. The relative importance of the several precautions and of the difficulties encountered can be expected to vary from species to species.

SUMMARY

A technique of quantitative differential agglutination of canine erythrocytes is presented. The dog isoantibody called anti-D in this laboratory has thus far proved most useful in this procedure, but at least three other dog isoantibodies can be used for differential agglutination.

The rate of elimination of two or more donor cell populations can therefore be measured simultaneously in a single recipient. The accuracy of the method described is approximately the same as that of the methods employed in differential agglutination of human erythrocytes.

The various applications of this procedure in transfusion studies in dogs are enumerated.

The two major difficulties encountered in studies involving differential agglutination of canine erythrocytes are the production of satisfactory antisera and the development of isoantibodies in the recipient following transfusion of initially compatible red cells. These difficulties are comparable to those encountered in experiments employing differential agglutination of human erythrocytes.

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POST-TRANSFUSION SURVIVAL OF FRESH AND STORED DOG ERYTHROCYTES MEASURED BY DIFFERENTIAL AGGLUTINATION

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THE purpose of this paper is to present data on the survival of dog red cells transfused immediately after withdrawal from the donor, and after storage at 4° C. for periods of four days and fifteen days. Measurements of survival of the transfused red corpuscles were made by the method of differential agglutination employing a potent dog isoagglutinin (anti-D).

The life span of erythrocytes in normal dogs has been determined by labeling a group of the animal's own red cells as they are formed and following ing the disappearance of these cells from the circulation.[1-4] Results obtained by these methods seem to be in good agreement, with periods of over one hundred days being demonstrated. This period is now generally accepted as the life span of erythrocytes in normal dogs. Normal *transfused* dog red cells were shown to survive for more than ninety days in an earlier experiment in this laboratory, but the method of differential agglutination of dog red cells (by canine anti-A serum) employed at that time was relatively crude.[5]

A recently developed technique of *quantitative* differential agglutination of dog erythrocytes[6] was used in the studies to be described in this paper.

METHODS

Twenty- to thirty-pound healthy mongrel dogs were chosen as recipients. All recipient dogs' erythrocytes were D-positive and all donor dogs' erythrocytes were D-negative.[7] After transfusion the red cells of the recipient were therefore agglutinable by anti-D dog serum. Careful typing of both donors and recipients with all known dog blood typing sera was carried out prior to the transfusions in order to minimize the possibility of immunizing the recipient against the donor's erythrocytes. Cross-matching of donor's red cells with recipient's plasma and of donor's plasma with recipient's red cells was carried out in small test tubes, utilizing fresh oxalated plasma and red cells suspended in fresh autogenous plasma. These tubes were incubated for fifteen minutes at room temperature and lightly centrifuged before examination. Indirect antiglobulin tests were then carried out on the red cells in each tube employed in the cross-matching procedure.[7] Only donors and recipients compatible by all these tests were chosen. Low unagglutinable cell counts were obtained on recipients prior to transfusion, and the donors' cells were shown not to react with the anti-D serum.

In an effort to minimize changes in the recipient dog's blood volume following these transfusions, blood was withdrawn from each recipient in an amount approximately equal to the volume of blood to be transfused. Bleeding was carried out either during the twenty-four hours prior to transfusion or immediately before transfusion. In recent experi-

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ments, we have bled the recipient of an amount equal to that to be transfused immediately before transfusion. Up to one-third of the recipient's blood volume can be exchanged by this method without apparent harm to the recipient.

TRANSFUSION OF FRESH BLOOD

Two recipients not previously transfused were utilized in this experiment. On the day before the first transfusion, each recipient was bled 300 ml. by puncture of the external jugular vein. On the day of the first transfusion another 300 ml. of blood was removed and 300 ml. of freshly drawn donor blood was given by rapid drip into a leg vein. Approximately twenty-four hours later, a second transfusion of 300 ml. of fresh donor blood was given in a similar manner without preliminary bleeding of the recipient. Three milliliters

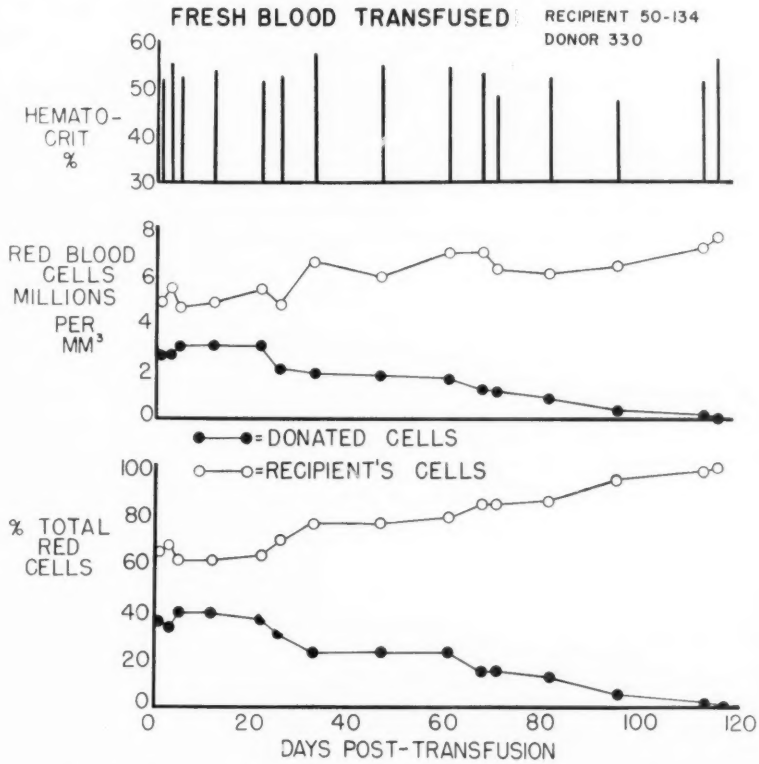

Fig. 1.—Post-transfusion survival of freshly drawn canine erythrocytes given to normal recipient dogs. Donor and recipient erythrocyte counts are expressed as number per cubic millimeter.

of saturated solution of sodium citrate were used as an anticoagulant for the donor blood for each transfusion. Transfusions were well tolerated and there was no evidence of hemolytic reaction. Immediately after each transfusion, both recipients showed mild instability of gait for approximately fifteen minutes. This disturbance was thought to be due to the rapid changes in blood volume and was not observed in subsequent experiments in which transfusions were administered more slowly.

The graphs of Figs. 1 and 2 reveal the manner in which the transfused red cells disappeared from the circulation of the recipients in these experiments. Survival periods of one hundred fourteen and one hundred six days were

demonstrated with nearly linear curves of destruction of the donated cells. The donor cell count showed greater variability during the first half of the survival period than during the second half. Unexplained variations in hematocrit were also encountered. These variations may reflect physiological changes in the animals' hematopoietic balance, or relative changes in plasma volume.

No attempts were made to determine the number of cells destroyed during and immediately after the transfusion by determining the recipient's blood volume and calculating the predicted rise in unagglutinable red cell count. Thus, these studies show only the rate of elimination of cells which survived the procedure of transfusion.

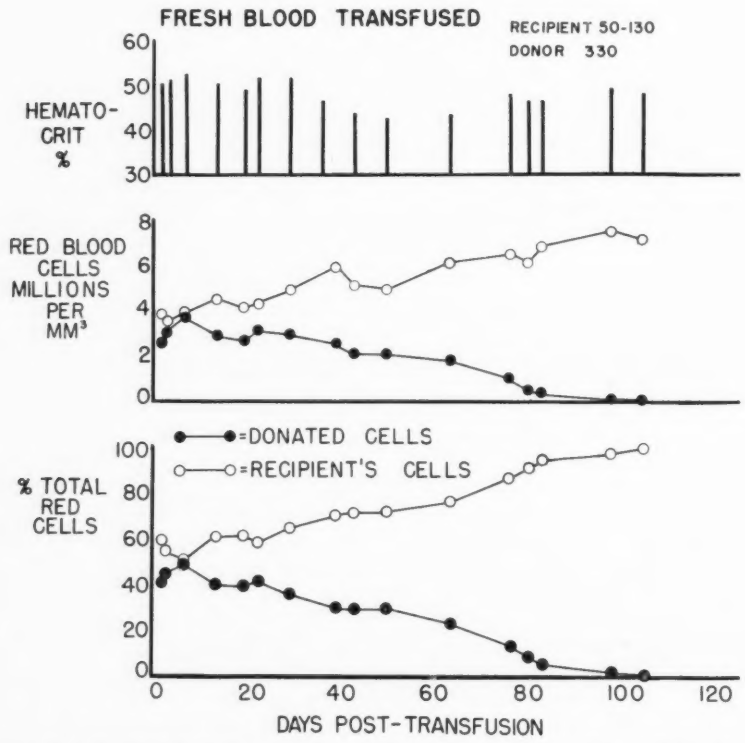

Fig. 2.—Post-transfusion survival of freshly drawn canine erythrocytes given to normal recipient dogs. Donor and recipient erythrocyte counts are expressed as number per cubic millimeter.

TRANSFUSION OF STORED BLOOD

The effect of storage in various anticoagulants upon the survival of dog red blood cells was investigated because of the need for such information in studies on the therapeutic effect of transfusions of stored blood to irradiated dogs.[8, 9] Preliminary in vitro studies of the effect of storage at 4° C. of sterile dog blood with concentrated sodium citrate or ACD solution (acid-citrate-dextrose blood preservative solution*) as anticoagu-

*Each 100 ml. of the ACD solution used in these experiments contained 2.30 Gm. of glucose, 2.06 Gm. of sodium citrate, and 0.75 Gm. of citric acid.

lants were carried out over a twenty-one day period. Determinations of hematocrit, pH, osmotic and mechanical fragility, and free plasma hemoglobin were made on the stored blood. These studies revealed that ACD solution in amounts equivalent to those used for storage of human blood was far superior to concentrated sodium citrate. The differences observed between these two anticoagulants were similar to those observed in studies of the storage of human blood.[10] For this reason, ACD solution, in vacuum flasks,* was chosen as the anticoagulant and preservative solution for the following experiments.

Two recipients not previously transfused were given blood obtained four days earlier from two D-negative donors. A third recipient that had been transfused once previously but whose serum contained no demonstrable isoantibodies was given blood obtained fifteen days earlier from a third D-negative donor. Unagglutinable cell counts were made before transfusion, and cross-matches were carried out as in the previous experiment. Blood was drawn under sterile conditions in 500 ml. units into 80 ml. of cold ACD solution. The

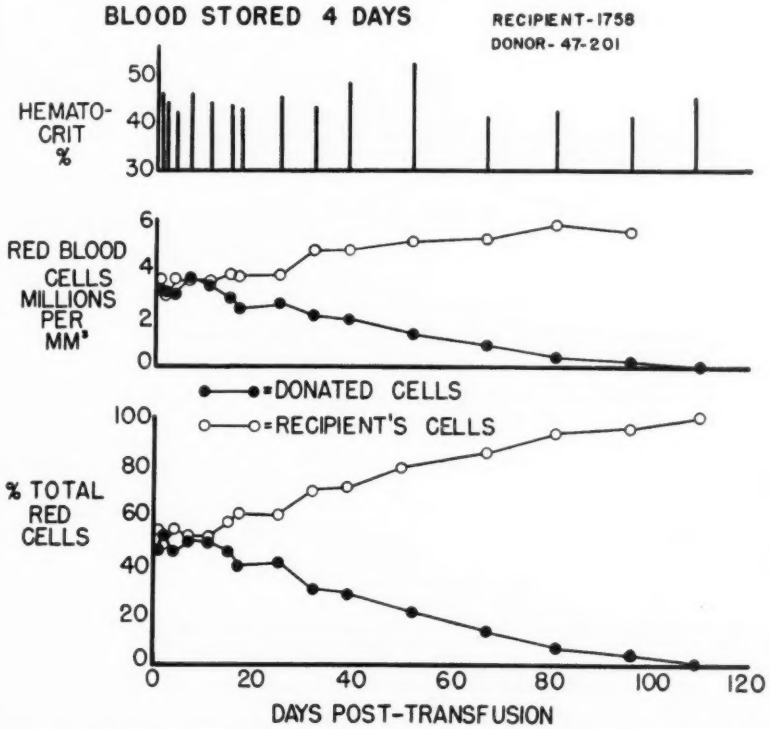

Fig. 3.—Post-transfusion survival of canine erythrocytes stored in ACD solution at 4° C. for four- and fifteen-day periods. Donor and recipient erythrocyte counts are expressed as number per cubic millimeter.

blood was stored at 4° C. Aerobic and anaerobic cultures obtained from the bottles prior to transfusion were sterile. The recipients that were to receive the four-day stored blood were bled a total of 440 ml. by two or three jugular punctures on the day before and immediately prior to transfusion. During the following twenty-four hours, transfusions were administered in amounts of 100 to 200 ml. given at a rapid rate into a hind leg vein through a standard blood administration set until an approximately equal amount (450 ml.) of blood had been replaced. The recipient that was given blood stored for fifteen days prior to transfusion was bled 250 ml. just before the transfusion of 250 ml. of the stored blood.

*The transfusion vacuum flasks, disposable donor sets, and blood administration sets used in these studies were manufactured by Baxter Laboratories, Inc., Morton Grove, Ill.

RESULTS

Figs. 3, 4, and 5 are graphs of the results obtained in these experiments. The first donor cell counts were made approximately twelve hours after the last of the transfusions of the 4-day-old blood. Destruction of transfused cells which might have occurred up to that time was therefore not determinable. Post-transfusion samples of plasma were free of hemoglobin. In the experiment with 15-day-old blood, the first determination of donor cell count was made twenty minutes after completion of the transfusion.

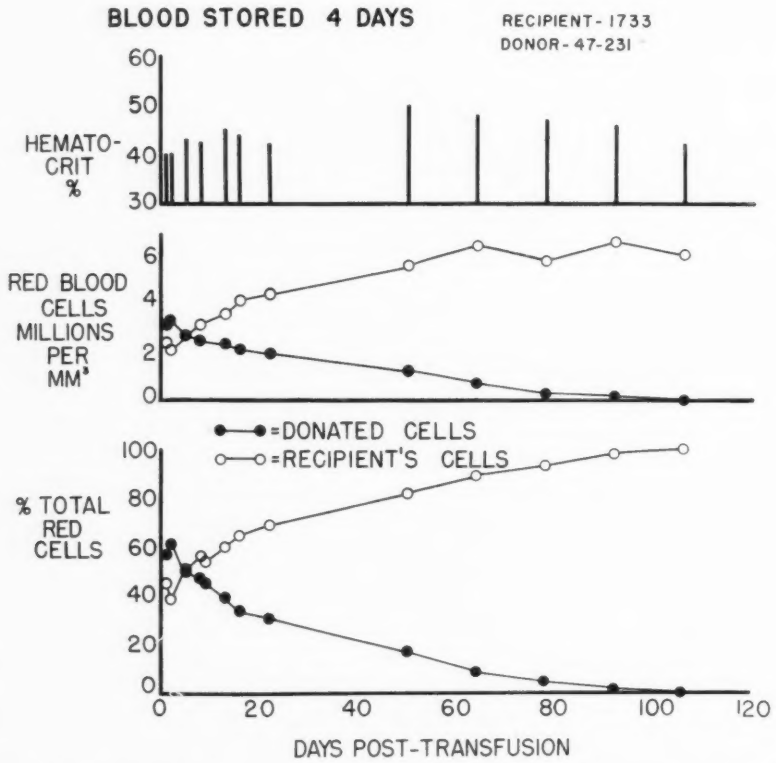

Fig. 4.—Post-transfusion survival of canine erythrocytes stored in ACD solution at 4° C. for four- and fifteen-day periods. Donor and recipient erythrocyte counts are expressed as number per cubic millimeter.

Essentially normal survival periods of approximately 108 and 100 days were obtained with 4-day-old blood. There was a period of approximately 20 days initially during which a slightly more rapid rate of destruction seemed to occur in the experiment shown in Fig. 4. The rate of disappearance of the donor cells then decreased and remained essentially normal.

The red cells that had been stored for fifteen days before transfusion survived for approximately eighty days after transfusion. Rather marked unexplained fluctuations of the recipient's hematocrit were observed during the first few days after transfusion.

DISCUSSION

The survival period of from one hundred to one hundred fourteen days for transfused normal dog red cells obtained in these studies is in excellent agreement with estimates of life span of dog red cells made by measuring the survival of a group of the animal's own labeled cells.[1-4] These studies demonstrate that transfused dog red cells survive a similar period of over one hundred days in normal recipient animals. The fact that this period is closely comparable to the normal red-cell survival period of man would seem to enhance the value of the dog in studying various problems involving hematopoietic equilibrium.

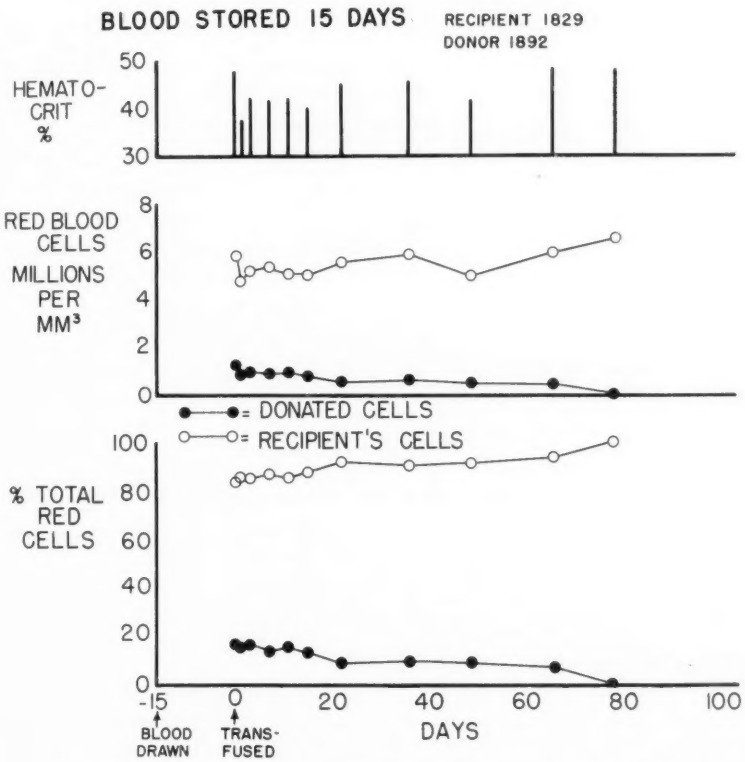

Fig. 5.—Post-transfusion survival of canine erythrocytes stored in ACD solution at 4° C. for four- and fifteen-day periods. Donor and recipient erythrocyte counts are expressed as number per cubic millimeter.

The results obtained in the studies of stored blood suggest that ACD is a satisfactory preservative solution for dog blood. These results seem comparable to those obtained in studies on storage of human blood.[11-14] It is therefore possible that the dog may be used to advantage for the study and development of more effective methods of blood preservation. Differences in composition of canine and human erythrocytes, e.g., potassium and sodium content, must be kept in mind, however, in evaluating results of such studies.

Similarly, it may be concluded that stored dog red blood cells survive effectively after transfusion if proper typing and cross-matching are carried out. We have recently demonstrated the practicality of a dog blood bank utilizing 250 ml. ACD flasks and transfusion equipment in conjunction with studies on transfusion therapy following x-irradiation injury.[8, 9] This may have a further field of usefulness for the veterinarian practice of transfusion, and in experimental surgery.

SUMMARY

Fresh normal transfused dog red cells, the survival of which was followed by the technique of differential agglutination, were shown to have a life span of well over one hundred days. This is in good agreement with estimates of the life span of dog red cells by other methods.

Dog blood stored at 4° C. in acid-citrate-dextrose blood preservative solution for periods of up to fifteen days survived satisfactorily when subsequently transfused to normal recipients.

The authors are indebted to the Clinical Branch of the University of Rochester Atomic Energy Project and especially to Dr. Frank Furth for assistance in carrying out several of these experiments.

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LABORATORY METHODS

A PHOTOGRAPHIC METHOD OF RECORDING RED CELL COUNTS

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THE purposes of this paper are to describe a method of recording red cell counts photographically and to present statistical evaluations of the accuracy of the method.

It is frequently necessary to perform large numbers of red cell counts during a relatively short period of time in the course of studies utilizing the technique of differential agglutination of erythrocytes. A variety of other experiments also involve large numbers of red cell counts. Attempts to conduct such experiments in this laboratory by employing large numbers of technicians of varying degrees of skill have frequently failed because of systematic errors in counting introduced by some of the less skilled persons. In an attempt to overcome this difficulty, a method has been developed for photographing the red cells in the counting chambers for later enumeration. This method permits the most skilled of the technicians to enumerate the cells from the photographic record at a leisurely rate. Fatigue is minimized by counting the cells over a longer period. Once a photographic record is prepared, moreover, counting may be postponed if other activities in the laboratory so demand. Since a permanent record is provided by the photographs, repeated recounting of the squares of the chambers can be carried out by the same or another person to minimize errors of enumeration.

MATERIALS AND METHODS

Suitably diluted erythrocyte suspensions in a variety of diluting fluids can be used. These can be prepared by any of the several methods commonly employed. We prefer the method of Daciel which utilizes relatively large volumes of whole blood and diluent.

The equipment for taking the hemocytometer chamber photographs and counting the red cells on the photographic record is shown in Fig. 1. It consists of a baseboard, upright, and arm assembly, a microscope equipped with 10× and 20× apochromatic objectives, 5× compensating eyepiece, and achromatic condenser, a microscope illuminator capable of focusing a filament image, and a Kine Exakta 35 mm. reflex camera, model II, equipped with a 50 mm. extension tube and appropriate adapter. The filament image of the lamp is first focused on the closed diaphragm of the microscope condenser, with the condenser in the uppermost position. The substage diaphragm is then opened to its maximum aperture. The filled counting chamber with a standard coverglass is placed on the stage and the ruled area located and brought into focus on the ground glass with the 10× objective. The 20× parfocal objective

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is then turned into place and after approximately two minutes have been allowed for settling of the cells, final focusing and setting of the field is carried out with the aid of the ground-glass magnifier built into this camera. A 10× objective and 10× eyepiece can be used if both 10× and 20× objectives are not available. Excellent results have also been obtained using achromatic objectives and Huyghenian eyepieces.

"Bright-line"* counting chambers have been most satisfactory, and critical focusing on the ruling of the chamber usually gives maximum contrast of the cells against the light gray background. Lowering the substage condenser 3 to 4 mm. may slightly increase the contrast. With this magnification, the photographed field includes six red cell counting subdivisions as shown in Fig. 2. The two symmetrically placed corner areas illustrated in section A of this figure were photographed on each side of the chamber. Five symmetrically placed squares (of the 6 squares photographed) on each frame were later counted.

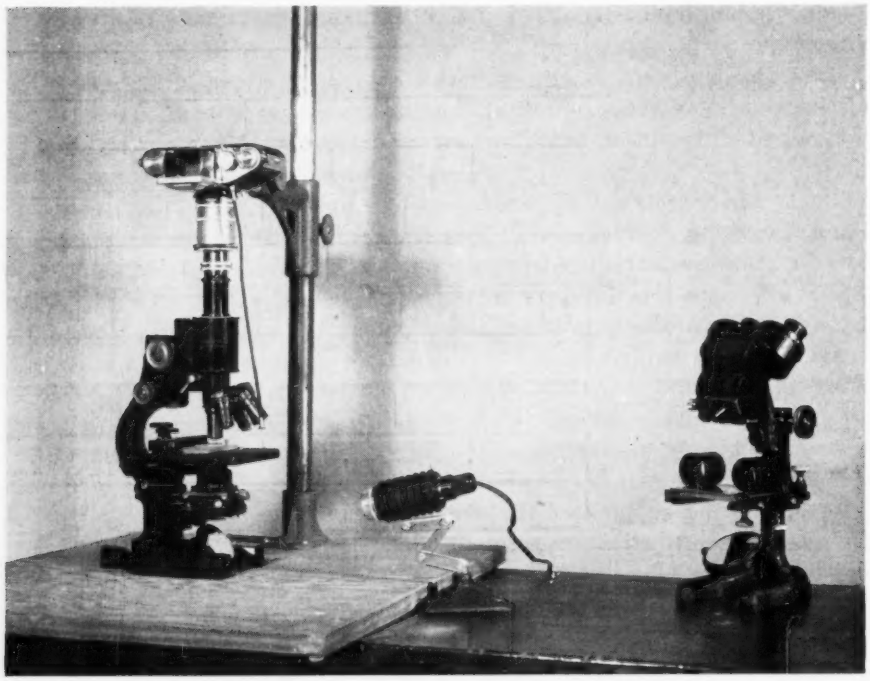


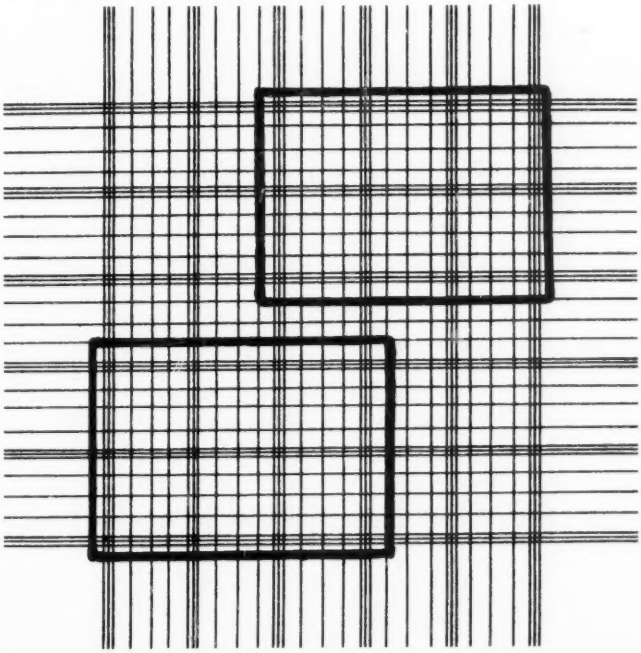
Fig. 1.—Equipment used for photographing and enumerating counting chamber photographs.

A cable release is used for all exposures. Kodak Linagraph, a high contrast, high speed orthochromatic 35 mm. bulk film, hand-loaded in casettes of 30 exposures each, is employed. Exposed film is developed for four minutes in fresh, high-energy x-ray developer, D-11, in tanks, with intermittent agitation. Developed film is fixed and then treated with a wetting agent before drying at room temperature. Individual frames are identified from a record of the order in which they were exposed and, when the film is dry, the frames are numbered with India ink on the emulsion surface corresponding with the record made during exposure. With this technique, exposures of 1/25 sec. are possible, and a negative of high density, good contrast, and satisfactory resolution is produced.

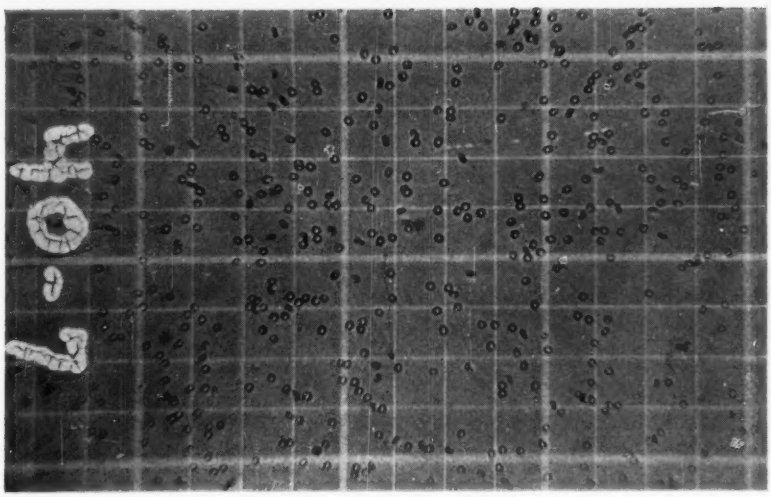
The cells can be enumerated directly from the negatives by several methods. The negatives may be projected in a darkened room by a microfilm projector and counts made from the screen, or the frames may be examined directly under low magnification. A binocular

*Made by American Optical Company, Buffalo, N. Y.

dissecting microscope illuminated by diffused transmitted light, as shown in Fig. 1, is most satisfactory for this purpose. Some type of guide for the film strip on the microscope stage is convenient. We prefer the latter method since it can be employed in a normally illuminated



A.



B.

Fig. 2.—*A*, Diagram of the central ruled area of American Optical Co. "Bright-line" counting chamber. The areas enclosed by the heavy lines are those photographed.

B, Print prepared from a photographic red cell counting negative. This area corresponds to those shown in *A*. The frame identifying number is on the left.

room and provides somewhat better definition. We have not found it advantageous or necessary to prepare enlarged prints of the negative images for counting purposes.

RESULTS

Although the rulings of the chamber can be brought into sharp focus on the ground glass, these lines are not as sharply resolved when examined on the negatives as when examined directly through the microscope. The rather coarse grain structure of the film contributes to the loss of resolution. Judgment as to whether a cell is or is not touching a line is therefore somewhat poorer on film strips. This is especially true with counts above 75 to 100 cells per red cell counting square. On the other hand, the error introduced by this difficulty may be offset by the ease of re-examination of the squares, and by permitting the counts to be made at a slow rate.

STATISTICAL STUDIES

Several experiments have been devised to compare this photographic red cell counting method with the direct visual microscopic counting procedure. In the first of these experiments a suspension of normal human red cells was made with 1 per cent formalin in 3 per cent sodium citrate solution[1] as a diluent. After direct transfer to a counting chamber the suspension contained approximately 50 cells in each red cell counting square. One side of a chamber was filled with this suspension. The red cells in the same 5 squares were then counted in one corner of the central ruled area by each of two well-trained technicians. Counts were recorded by individual squares independently by each person. The photographic apparatus was then swung into position and the area in which the counts were made was photographed. This was repeated 31 times. The films were developed, identified by serial number, and the same areas recounted independently by the two technicians.

The results of this experiment have been analyzed by the technique of analysis of variance. The average of the direct visual microscopic counts for both technicians was 269 cells per 5 squares, with a standard deviation of 2.2. There was no evidence of a systematic difference in counting between the two technicians with this method. The average of the photographic counts for both technicians was 266 cells per 5 squares, with a standard deviation of 5.4. Again there was no evidence of a systematic counting difference between the two technicians. The probability that the difference between these averages could be observed, if in fact there were no difference between the values, is considerably less than 0.01. Thus it may be concluded that it is highly probable that a small but real difference exists between the results of the two methods, with the photographic method giving results of the order of 1 per cent lower than the direct microscopic method of counting at a level of approximately 270 cells per 5 red cell counting squares. It will also be seen that there is some increased variability of the results of the photographic method. This suggests, but does not clearly demonstrate, that the direct microscopic method of counting may give results more closely approximating the "true" values than does the photographic method.

The second experiment was carried out with only one technician, skilled in both the photographic and direct methods of red cell counting. A series of red cell counts by the method of Dacie[1] were carried out on a normal dog over a period of several months. The same 5 square areas of the counting chamber were counted directly and by the photographic method on 30 determinations. Counts of approximately 550 cells in 5 squares were consistently obtained. It was found that counts by the direct method of counting averaged 2.3 per cent higher than those obtained by the photographic method, with a standard deviation of the difference of ±3.4 per cent. The range of ±2 S.D. of from −4.5 per cent to +9.1 per cent was in close agreement with the observed. A "t" test of the observed difference yields "t" = 4.26. This would indicate that the probability is very high that there is again a real but small difference in the results obtained by the two methods.

Recounting of the same chamber photographs at variable intervals by the same technician has been done on several occasions. In general, excellent agreement is obtained at lower levels of count, up to about 300 cells per 5 squares. With higher counts, some increase in variability of recounts occurs, probably due to variable judgment concerning the counting of cells near lines. This may be no greater than that which might be seen if the same areas were to be recounted directly under the microscope. Equival it data regarding variability upon recounting by direct visual enumeration cannot be developed.

DISCUSSION

There is little question that the direct and photographic red cell counting methods do not give identical results. Hirsch and co-workers[2] presented a similar photographic method in 1950 and concluded that its accuracy was less than that of direct visual counting. On the basis of the present data, it is impossible to determine absolutely which method gives a closer approximation to the "true" count. It can only be inferred that the direct visual method may give more accurate results. Results obtained by either method are probably strongly dependent upon variable, poorly understood, and poorly controlled psychological factors affecting the technician. An elaborate experimental and statistical evaluation of both techniques would be required to evaluate the effect of these factors for any one technician in a given experimental situation. It is obvious that there is a real error of enumeration present in both methods. This error may vary with the absolute level of the count. The difference between the two methods is of a relatively small order, however, regardless of which method may be the more accurate. Thus, we believe the method is sufficiently accurate to be useful in many experiments involving red cell counting. Furthermore, in experiments requiring large numbers of red cell counts, it is possible that with direct counting methods errors introduced by fatigue of technicians may be much greater than any inherent errors of the photographic method, making the latter method clearly the one of choice in such situations. It should be emphasized again that this method is not conservative of time, effort, or expense, or necessarily more accurate than the

direct method of enumeration. It offers a real advantage, however, in situations requiring the recording of large numbers of counts in a short period of time.

SUMMARY

A photographic method of recording red cell counts is described.

Erythrocyte counts carried out by this method are slightly lower and slightly more variable than those obtained by direct visual microscopic enumeration. These differences have been analyzed statistically.

The photographic method is advantageous in circumstances requiring the recording of many erythrocyte counts in a short period of time.

The authors are indebted to Dr. S. L. Crump and Dr. Arthur Dutton, Statistics Section, Atomic Energy Project in the University of Rochester, for aid in the statistical analyses.

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RAPID DETERMINATION OF URINARY SODIUM

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THE clinical practice of estimating sodium excretion by measuring chloride excretion is not always justified. However, for want of a simple method for urinary sodium, chloride determinations are still used extensively in many instances where information concerning sodium excretion is required.

A method is here presented for the measurement of urinary sodium for which only one reagent is necessary, and by which four determinations can be made in fifteen minutes. No particular experience or skill is needed.

METHOD

Reagents.—Uranyl zinc acetate solution as prepared by Albanese and Lein.[1] Their directions are: "10 Gm. of uranyl acetate were dissolved in 50 ml. of boiling water containing 2 ml. of glacial acetic acid. In another flask 30 Gm. of zinc acetate were dissolved in 50 ml. of boiling water containing 1 ml. of glacial acetic acid. The boiling solutions were mixed and again heated just to boiling. After standing overnight at room temperature, the resultant solution was filtered by gravity and mixed with an equal volume of 95 per cent ethanol. This solution was refrigerated at 4° C. for forty-eight hours and then filtered by gravity for use."

The same reagent reduced to one-third strength with water.

Apparatus.—Special centrifuge tubes with elongated calibrated tips. "Urine Protein, Shevky-Stafford, Kimble Glass," were first used, although it was necessary to reject some

TABLE I. COMPARISON OF RESULTS

SAMPLE	K MEQ./L. FLAME PHOT.	CL. G.P.L. AS NaCl	Na G.P.L. AS NaCl CHEM. METH.	Na G.P.L. AS NaCl FLAME PHOT.	DIFFERENCE CHEM.—F.P.
1	4	1.0	1.0	1.0	0
2	16	1.7	1.3	1.5	−.2
3	39	2.5	2.6	2.6	0
4	36	3.4	2.9	3.0	−.1
5	71	3.3	3.5	3.3	+.2
6	68	3.8	4.5	4.7	−.2
7	91	5.5	4.8	4.6	+.2
8	60	7.1	7.9	7.8	+.1
9	45	8.0	9.5	9.3	+.2
10	53	8.3	9.8	9.9	−.1
11	6	1.5	1.0	0.7	+.3
12	46	0.7	<0.1	.05	0
13	57	3.1	2.7	2.8	−.1
14	72	3.1	2.3	2.2	+.1
15	32	3.8	3.1	3.3	−.2
16	28	4.9	4.4	4.1	+.3
17	16	5.3	5.4	5.3	+.1
18	54		8.0	8.0	0
19	24	4.0	4.2	4.1	+.1
20	36	8.7	9.9	9.7	+.2

From the Biochemical Laboratory, St. Michael's Hospital, Toronto.
Received for publication, April 9, 1951.

due to inaccurate zero points. Later a more suitable tube was obtained locally,* in which the calibrated tip, containing a volume of 0.2 ml., was one inch long. See Fig. 1.

Procedure.—To 1.0 ml. of urine in a test tube add 1.5 ml. of dilute sodium reagent. Mix and centrifuge for three minutes.

To 3.0 ml. of sodium reagent in a special centrifuge tube add 1.0 ml. of supernatant fluid and mix immediately with a fine glass stirring rod. This step is carried out with the rack of centrifuge tubes containing the reagent, sitting in water at 22° C. Let stand about two minutes. Centrifuge at 2,000 r.p.m. for seven minutes. Read the volume of the precipitate.

Fig. 1.—Special centrifuge tube, four-fifths actual size.

Calibration.—A standard curve was obtained by taking the average of six, each one of which was obtained in the following way. About 350 ml. of urine, which contained no sodium or less than 5 meq. per liter by flame photometer, were obtained from the hospital ward. After dividing into 50.0 ml. aliquots, measured amounts of a 5.00 per cent sodium chloride solution were added and the procedure carried out on each specimen as well as on the original sample. The curve, which is not quite linear, is reproduced in Fig. 2.

RESULTS

Twenty urines from hospital patients were selected by the use of approximate chloride determinations so that the range from 0 to 10 Gm. per liter of sodium chloride was adequately represented at all levels. The Beckman Flame Photometer was used to make the comparison. The results are given in Table I. The figures for potassium, as determined by the photometer, are also given to show the lack of interference by this ion. The chloride values were obtained by the method of Wilson and Ball.

*O. H. Johns Glass Co. Ltd., Toronto, Ontario.

DISCUSSION

Interference was to be expected from three substances: protein, phosphate, and potassium. The first two are conveniently precipitated by the 1.5 ml. of dilute uranyl zinc acetate reagent. The supernatant fluid obtained has always

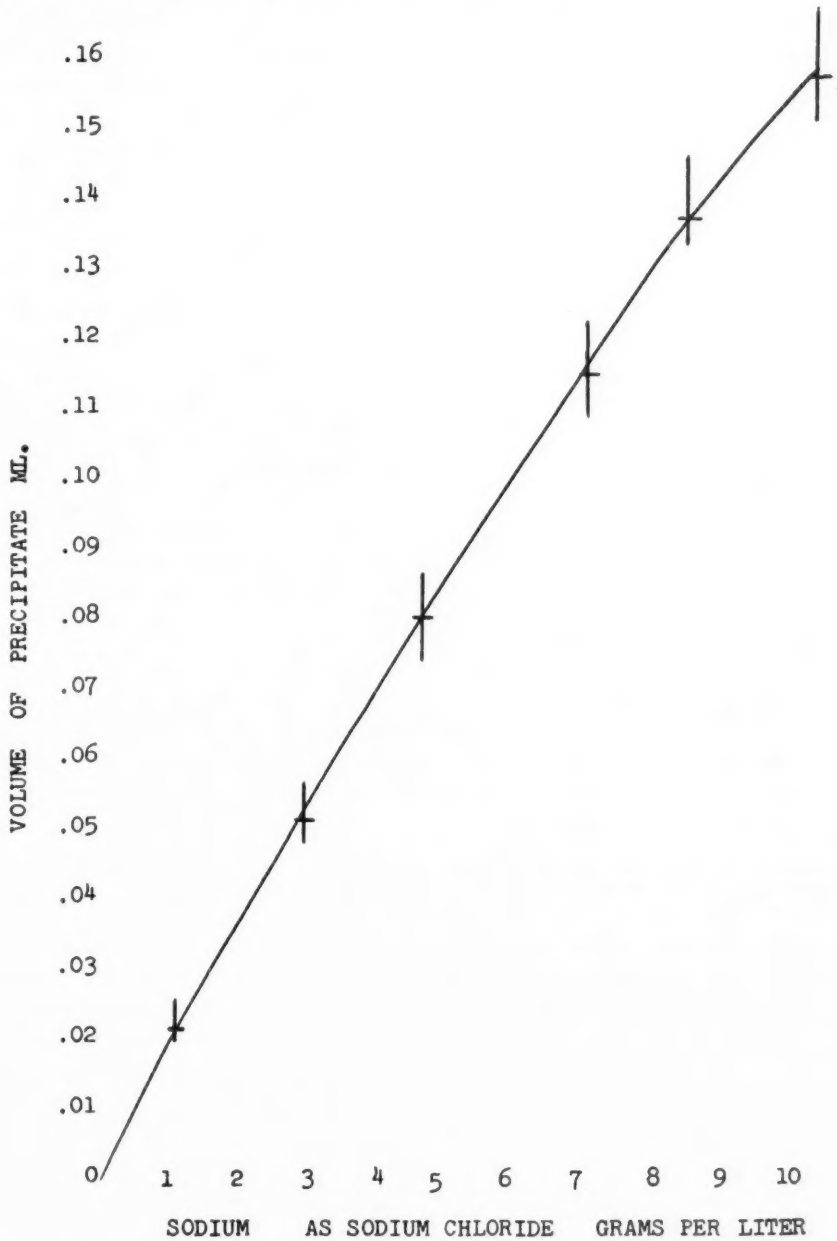

Fig. 2.—The standard curve. Each point represents the average of six determinations. The vertical lines represent the range of the readings.

been crystal clear. The potassium salt does not come down in the second step as long as the procedure is carried out at room temperature. Added potassium, equivalent to 100 meq. per liter, did not cause appreciable error unless the mixture was refrigerated. Finally 22° C. was chosen and adhered to, when it was noticed that warm weather altered room temperature enough to appreciably reduce the volume of the precipitate obtained.

It was found necessary to use urine solutions of sodium chloride for the standard curve because ordinary saline standards gave precipitate volumes which were slightly, but persistently, lower than those obtained from known urine solutions. This phenomenon was true, in spite of the fact that before salt was added to the urine no measurable amount of precipitate was formed by the procedure. The standard curve is, of course, a function of the diameter of the centrifuge head, the speed, and the time of centrifugation. In this work an International Type SB I was used throughout at 2,000 r.p.m. for seven minutes.

The speed and simplicity of a method such as this, which is intended for everyday clinical use, is more important than an accuracy of 1 or 2 per cent. It has been used in this laboratory for two years, is sufficiently accurate, i.e., to the nearest half gram per liter, and in our hands is faster than the flame photometer. Most technicians are capable of carrying out the test after being shown a single time. The results are very objective, always an advantage in clinical laboratory work.

The present range from zero to ten grams per liter calculated as NaCl, is seldom exceeded in hospital practice. It does not, therefore, seem worth while to increase the amount of rather expensive reagent used. At higher levels a urine dilution may be used.

SUMMARY

A simple and rapid method is presented for the estimation of sodium in urine in amounts up to 10 Gm. per liter calculated as NaCl. Only one reagent is used. Comparison of results with values obtained by the flame photometer shows satisfactory agreement. The method is faster than the flame photometer and demands less in technical skill.

Most of the work required to produce the calibration curve and the table of results was carried out by Miss Rickie McCallion and Miss Inge Frandsen. Their assistance is gratefully acknowledged.

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A RAPID MODIFICATION OF THE BRUCINE METHOD FOR DETERMINATION OF NITRATES IN BIOLOGICAL FLUIDS

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WIDESPREAD interest in nitrates has been accumulating over the past several years. Their diuretic action is well known,[1] and has been reiterated recently by Hiatt.[2] Nitrates have been used as loading solutes in experiments on osmotic diuresis in dogs.[3a, b, c] Their role in electrolyte metabolism was demonstrated by Hiatt, who showed that nitrates could replace 40 per cent of chlorides in the dog without significant ill effects.[4] Moreover, pediatric literature has called attention to the methemoglobinemia following the ingestion by infants of well water contaminated with nitrates.[5]

It appeared of interest to develop an accurate, sensitive, and rapid method for determination of nitrates in biological fluids.†

The use of brucine‡ in a colorimetric determination of nitrates was suggested by Winkler.[6] Modifications of the brucine method were developed by Snell for use in meat extracts,[7] by Wolf for determinations in plant and soil extracts,[8] and by Noll for the nitrate in boiler water.[9] None of the foregoing methods has been found satisfactory, in our hands, for determination of nitrates in plasma and urine. The present method provides means for a rapid determination with a sufficient degree of reproducibility and sensitivity. An alternate procedure to be used for solutions containing nitrites as well as nitrates is also presented.

METHOD A

DETERMINATION OF NITRATES IN THE ABSENCE OF NITRITES

Reagents.—Nitrate standard—5 Gm. of KNO_3 are carefully weighed and dissolved in one liter of distilled water. This will contain 3.07 mg. of NO_3 per milliliter or approximately 50 milliequivalents of NO_3 per liter.

Brucine-H_2SO_4 Reagent—Pure brucine alkaloid is "dissolved" in concentrated (18 molar) reagent grade sulfuric acid. One milligram of brucine should be present for every milliliter of H_2SO_4. The quantity needed for a single set of determinations is prepared *immediately* prior to use.

5 per cent Mercuric chloride

1 per cent Sodium carbonate

Preparation of Protein-Free Filtrate From Plasma.—0.5 ml. of plasma containing nitrate is placed in a 25 ml. volumetric flask. 5 ml. of distilled water and 0.5 ml. of 1 per cent Na_2CO_3 are added with mixing between each addition. 2.5 ml. of 5 per cent $HgCl_2$ is then added and the contents made up to 25 ml. with distilled water. Shake well, centrifuge, and remove 1 to 2 ml. of the clear supernatant fluid for analysis.

Preparation of Urine.—Nitrate containing urines are diluted with distilled water to provide a concentration of 5 to 30 micrograms of nitrate per milliliter and 1 to 2 ml. aliquots of the dilutions are taken for analysis. Urinary urea, at concentrations below 500 mg. per 100 ml., will not interfere with the reaction.

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†Modifications suggested herein render the method suitable for determinations of nitrate with or without nitrites, in well water, boiler water, and other solutions.

‡The use of diphenylbenzidine, diphenylamine, or of ferrous chloride and hydrochloric acid in the determination of nitrates has been described by Whelan.[13]

Calibration of Method Using Water Standards.—Dilutions of the standard KNO_3 solution are prepared to allow for a range of 5 to 30 micrograms of NO_3 per sample tube. Aliquots are treated in the manner described below and a calibration curve, based on the observed optical density per microgram of nitrate (i.e., the "K" value) versus the optical density readings, is constructed.

Procedure.—Aliquots of unknown or standard solutions plus water are pipetted into 25 ml. test tubes. The total volume of both should be exactly 2 ml. Add 5 ml. of the brucine-H_2SO_4 reagent rapidly down the side of the tube and thoroughly mix with a flat-bottomed rod. Allow each tube to stand exactly five minutes prior to the next step. The five-minute interval pertains to each individual tube, not to the whole set. Rapidly add 5 ml. of water and mix thoroughly. Cool for ten minutes in a water bath at room temperature. Read at a wave length of 440 Å on the Coleman Junior spectrophotometer against distilled water.

When analyzing plasma filtrates or urines, it is important to perform simultaneous analyses on water blanks (2 ml. of distilled water plus all reagents) and appropriate standard solutions.

Calculations.—A "K" value for each unknown is determined by reference to the curve in which "K's" obtained from standard solutions have been plotted against their respective densities. The concentration of the unknown sample is obtained by the formula:

$$\text{Micrograms/ml.} = \frac{\text{density} - \text{blank}}{\text{K}} \times \frac{\text{dilution}}{\text{aliquot}}$$

Results.—Shown in Table I, A are optical density readings with calculated "K" values for standard KNO_3 solutions over a range of 5 to 30 micrograms of NO_3 per sample tube. Each value represents the mean of five to ten separate determinations. The analytical error between duplicate determinations varied from 2 to 4 per cent. Variations from one set of determinations to another, apparently caused by minor variations in the brucine reagent, were no greater than 10 per cent.

It may be seen that optical density* increased in almost direct proportion to the amount of nitrate. "K" values, or density per microgram, indicate a relative and systematic reduction of color development in the reaction at high levels of nitrate concentration. Such a reduction may be due in part to the use of a Coleman Junior spectrophotometer, wherein the optical system does not provide pure monochromatic light.

The yellow color formed by the brucine reagent under the conditions described was stable for the first thirty minutes after cooling and began to

TABLE I. OPTICAL DENSITIES AND CALCULATED "K" VALUES OBTAINED WITH STANDARD SOLUTIONS OF NITRATE

MICROGRAMS NO_3	DENSITY-BLANK	"K"
Method A		
5.09	0.123	0.0242
10.17	0.236	0.0232
15.26	0.334	0.0219
20.34	0.439	0.0216
25.45	0.515	0.0202
30.52	0.628	0.0206
Method B		
10.17	0.110	0.01082
15.26	0.145	0.0095
30.52	0.245	0.00804
40.68	0.304	0.00746
45.76	0.334	0.00731
61.04	0.430	0.00705

*Density values for reagent blanks, not presented in the tables, were of the order of 0.010 to 0.020.

TABLE II. STABILITY OF COLOR DEVELOPMENT WITH TIME AFTER COMPLETION OF REACTION

NO_3 PRESENT	DENSITY READINGS AFTER COOLING TIME			
	0 MIN.	30 MIN.	60 MIN.	12 HOURS
Method A				
10.2	0.244	0.244	0.242	–
15.3	0.349	0.349	0.341	–
20.3	0.452	0.455	0.438	–
Method B				
10.17	0.128	0.128	0.125	0.124
20.34	0.186	0.185	0.184	0.180
30.52	0.283	0.282	0.283	0.278
40.68	0.315	0.312	0.314	0.303

fade significantly by the end of one hour. In Table II, *A* are values representing density readings versus time at three levels of concentration. Fading appeared somewhat greater with increasing concentrations of NO_3.

To determine the efficiency of the method for nitrate-containing plasma, gravimetrically prepared amounts of standard KNO_3 solution were added to plasma, prior to the preparation of filtrates. Concentrations selected were approximately those found in dog plasma after nitrate loading at levels of 5 to 40 millimoles per liter. Table III, *A* illustrates data obtained on recovery of nitrates from the plasma. Analytical recoveries were of the order of 92 to 106 per cent.

Under conditions of the method, as shown in Table IV, *A*, color was formed by nitrites as well as by nitrates. This observation is of little significance in many applications of the method. For example, urine and plasma from dogs after intravenous nitrate loading contained no determinable nitrite.[10]

Urea may also interfere with the nitrate determination described; but its action seems to be negligible at the high dilution of plasma and urine employed. No interference was found at levels up to 500 micrograms of urea per sample tube.

TABLE III. RECOVERY OF NITRATE FROM PLASMA

PLASMA CONC. AFTER ADDITION OF NO_3	MICROGRAMS NO_3 PRESENT IN ALIQUOT	MICROGRAMS NO_3 FOUND	PER CENT RECOVERY
Method A			
508 microgm./ml.	10.17	10.07	99
1017 microgm./ml.	15.26	14.02	92
	10.17	10.8	106
	20.31	20.3	100
Method B			
1526 microgm./ml.	15.26	15.15	99.3
	20.34	20.3	100
	30.52	30.13	99.2
	45.76	45.6	99.5

METHOD B

DETERMINATION OF NITRATES IN THE PRESENCE OF NITRITES

If desired, nitrites and nitrates may be analyzed quantitatively in the presence of each other. For nitrate alone, add 0.5 ml. of an ammonium sulfamate solution, containing 1 Gm. of ammonium sulfamate per 100 ml. water, to a 1.5 ml. aliquot of the unknown, and continue stepwise as recommended under procedure for Method A above. Ammonium sulfamate is added in order to remove nitrites.[11] Because of a reduction in sensitivity, the sulfamate modification required construction of a new calibration curve.

TABLE IV. EFFECT OF INTERFERING SUBSTANCE ADDED TO REACTION MIXTURE ON COLOR FORMATION

SUBSTANCE ADDED	CONCENTRATION OF ADDED SUBSTANCE (MICROGRAMS)	NO_3 PRESENT (MICROGRAMS)	OPTICAL DENSITY (MINUS BLANK)
Method A			
Urea	0	10.17	0.215
	50	10.17	0.216
	500	10.17	0.217
	5000	10.17	0.184
Nitrite	0	10.17	0.215
	10.2	10.17	0.473
	0	15.26	0.331
	15.3	0	0.369
	15.3	15.26	0.595
	0	20.34	0.434
	20.3	0	0.485
Method B			
Urea	0	10.17	0.086
	50	10.17	0.084
Nitrite	150	0	0.000
	0	15.3	0.134
	100	15.3	0.135
	0	20.3	0.173
	150	20.3	0.174
	0	30.5	0.245
	150	30.5	0.241
	0	61.0	0.430
	150	61.0	0.426

The range of nitrates recommended, with the corresponding optical densities and calculated "K" values, is shown in Table I, *B;* color stability in Table II, *B;* and per cent recovery of nitrate added to plasma in Table III, *B*. Table IV, *B* illustrates the absence of significant interference in color formation when nitrites or urea are added to the reaction mixture.

For determination of nitrites alone, a diazotization method such as that of Bratton and Marshall[11] may be employed after appropriate modification (i.e., measured amounts of nitrites are mixed with a fixed excess of para-aminobenzoic acid reagent). Experiments in the authors' laboratory had demonstrated that nitrates do not interfere with the diazotization reaction.

DISCUSSION

The development of a yellow color with brucine in the presence of nitrates and concentrated sulfuric acid may be explained by analogy to an oxidation-reduction reaction described by Kolthoff.[12] Using diphenylbenzidine as an indicator, he found that nitrates in the presence of excess acid were reduced to nitrogen oxide, and a blue color resulted. The color was attributed to the formation of an oxidation product of diphenylbenzidine. He was unable to quantitate the color according to Beer's Law because of a cycle in the reaction mixture in which nitrogen oxide was partly oxidized by oxygen from the air to nitrite, which again reacted with the indicator, thereby producing an excess of color. Data reported herein suggest that brucine, in the presence of nitrates, reacts in a manner similar to diphenylbenzidine.

That H^+ per se is an important factor in the "brucine-nitrate" color complex is borne out by experiments wherein color was obtained when concentrated hydrochloric acid was substituted for sulfuric acid, if sufficient external heat was applied. On the other hand, decreasing the molarity of the sulfuric acid to 10.5 resulted in no color production even after as much as thirty min-

utes in a boiling water bath. With 15M H_2SO_4, color production was satisfactory, but slow in development. The addition of 18M (concentrated) acid to the aqueous mixture, with the spontaneous heat evolution, produces immediate high levels of color formation.

The addition of the final volume of water serves, when nitrate is being determined, to so dilute the acid that the reaction ceases.

Although the method employing ammonium sulfamate is less sensitive than Method A, it has obvious application in determination of nitrates in the presence of nitrites. Data of Table IV, A indicate that nitrites, added to nitrates, increase optical density in a nonproportionate manner. Therefore, subtraction of the value for nitrites determined separately[11] is not justified.

CONCLUSIONS

A method for the rapid spectrophotometric determination of nitrates in urine and plasma has been presented. Data indicate that Method A is sufficiently sensitive to detect nitrates in amounts of 5 to 30 micrograms, and Method B in amounts of 10 to 60 micrograms per aliquot. Method B may be employed for determination of nitrates in the presence of nitrites. Close proportionality between optical density and amount of nitrate was obtained. The analytical error was of the order of 2 to 4 per cent between duplicate or triplicate determinations. The nature of the color complex formed by brucine in the presence of nitrates has been discussed.

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IMPROVED TOTAL BASE DETERMINATION IN SERUM, URINE, AND TISSUE ASH BY ION EXCHANGE

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WITH THE TECHNICAL ASSISTANCE OF H. BLOEMENDAL

INTRODUCTION

THE total base content of serum can be determined by the ion exchange method worked out by Polis and Reinhold (1944). In this method a known amount of serum is absorbed in a column of a cation exchange resin (hydrogen-form). The resin is eluted with water and the eluate, containing an amount of hydrogen ions equivalent to the total base content, is titrated with alkali to a pH 7.4 against phenol red or bromothymol blue after removal of carbon dioxide by aerating. In view of our own experiences with this method we refined the procedure by including blank determinations, by a closer control of the percolation rate, by diluting the samples before introduction to the columns, by employing other exchange resins, and by using the same columns for a large number of determinations instead of discarding them after each determination.

Gorter and Van Royen (1948), employing a sulfonated coal for the same determination, collect the effluent in an excess of sodium hydroxide and titrate the excess with hydrochloric acid against bromothymol blue. They claim to determine in this way also the base bound to the serum bicarbonate. These are not included in Polis and Reinhold's procedure. A combination of the methods of Polis and Reinhold, and of Gorter and Van Royen would enable us to determine both the total base and the bicarbonate content of the serum by ion exchange technique. This turned out to be impossible, however, due to considerable and varying losses of carbon dioxide, even with special precautions. In a recent publication, which reached us after finishing our own experiments, Süllmann (1952) describes the use of an anion exchange resin in order to include the cations bound to bicarbonate in the total base determination. In the case of serum, however, the proteins affect the titration of the effluent. No suitable deproteinization method having been found so far, this method cannot be used for serum.

Finally, we worked out methods for the determination of the total base content of urine and tissues.

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MATERIALS AND METHODS

In our experiments we employed the following ion exchange resins: Dusarit,* a sulfonated coal, and Amberlite IR 105 – H and IR 120 – H†, sulfonic acid cation exchange resins in hydrogen form.

The columns had a diameter of 10 mm. and were 140 mm. long. At the lower end they had a stopcock with a capillary tip, and, at the upper end a cup 50 mm. long and 40 mm. in diameter. We used resin columns with a height of 130 mm., resting on a small plug of glasswool. The resin column is always kept immersed by water.

The following procedure was finally adopted for serum. Titrations are carried out with 0.02N NaOH in a 2 ml. Bang burette against bromothymol blue. Before titration all solutions are heated to the boiling point in order to eliminate the dissolved carbon dioxide. The column is washed with distilled water until a 10 ml. sample of the effluent can be neutralized by 0.03 ml. or less of 0.02N NaOH. After completion of the washing the water level in the column is brought at a mark 10 mm. above the resin surface. Then 0.15 ml. of serum is diluted with distilled water to 3 milliliters. This solution is added quantitatively and percolated at a rate of 0.8 ml. per minute (1 drop in 2 seconds) until the water level reaches the mark; the tube containing the diluted serum is rinsed with 5 ml. of distilled water and this is added to the column, followed by 10 and 15 ml. of distilled water. The effluent is collected in a 50 ml. Erlenmeyer flask, heated to the boiling point, and titrated. Again 10 ml. of water are percolated and titrated. The blank values obtained before and after percolation of the diluted serum are used to correct the titration value for the serum sample. The total base content of the serum sample is calculated by comparison with the values obtained for some standards consisting of 0.15 ml. of 0.150N NaCl. Each column can be used at least 30 times without regeneration or changing of the resin.

Urine has to be neutralized first. An amount of urine containing about 30 microequivalents of base (about 50 microliters in the case of rat urine) is neutralized after the addition of one drop of bromothymol blue. In the case of acid urine it is neutralized with 0.02N NaOH and the amount used is recorded, while alkaline urine is neutralized with 0.02N HCl without estimation of the amount used. After dilution to 3 ml. the solution obtained is treated as described for serum. In order to calculate the total base content of the urine the amount of NaOH added is subtracted from the titration value of the effluent.

The determination of the total base content of tissue ash can be carried out as follows. A tissue sample of about 1 Gm. is dried overnight in a crucible (if possible platinum) at 105° C., and is subsequently ashed in an electric furnace at 500 to 550° C. for five to seven hours. After cooling in a desiccator, 4 ml. of 0.075N HCl are added, and the ash, still containing a few particles of carbon, is dissolved by stirring with a glass rod having a flat end. The crucible is left for one to two hours in a desiccator containing 0.075N HCl. A 0.25 ml. aliquot of the solution is then transferred to a small test tube and neutralized with 0.02N NaOH against bromothymol blue. After dilution to 3 ml. the solution is treated as described for serum. In the calculation of the total base content of the tissue the same procedure as for urine is followed.

RESULTS

Choice of Resin.—With Dusarit we obtained high and varying blank values. We therefore abandoned this substance in our further experiments. The Amberlite cation exchange resins IR 105 and IR 120, after sufficient rinsing, gave rather constant and low blank values. Generally, when the columns were used for the first time or after standing overnight, rinsing with about 100 ml. of distilled water at a drop rate of 1 to 2 drops per second sufficed to give a blank value of 0.03 ml. of 0.02 N NaOH or less. This value did not change markedly during

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†Rohm and Haas Company, the Resinous Products Division, Philadelphia, Pa.

successive determinations. Ultimately, we chose the IR 120 resin which has double the capacity of the IR 105 resin and does not absorb iron irreversibly like the latter.

Completeness of Exchange.—We determined the recovery for Na^+, K^+, and Ca^{++}, with both the IR 105 and IR 120 resins, using 0.15 ml. samples of 0.15N chloride solutions. The results are given in Table I. In each case the average of ten determinations is given with the standard error of the mean. A percolation rate of 0.8 ml./min. (1 drop per 2 sec.) was employed in all determinations.

TABLE I.

SOLUTION	PER CENT RECOVERY	
	IR 105	IR 120
NaCl 0.15N	99.8 (± 0.2)	98.8 (± 0.2)
KCl 0.15N	99.3 (± 0.3)	98.9 (± 0.3)
$CaCl_2$ 0.15N	99.5 (± 0.6)	100.5 (± 0.7)

These recovery determinations for pure solutions were supplemented by flame photometric estimations of the Na^+ and K^+ concentrations in a number of effluents from serum samples using the IR 120 resin. The average loss of Na^+ for 20 determinations was equivalent to 1.9 (±0.3) meq. per liter and for K^+ it was 0.2 (±0.1) meq. per liter. So the total loss was 2.1 meq. per liter which is about 1.4 per cent of the total base content of the serum sample. No calcium could be detected in the effluents.

In order to obtain complete exchange, we found that the samples have to be diluted to a total base concentration of 0.005 to 0.01N before washing them through the column. Furthermore, we found that percolation at rates of 3 to 6 ml. per minute (as employed by Gorter and Van Royen) gave results that were about 3 per cent lower than those at a percolation rate of 0.8 ml. per minute (1 drop per 2 seconds). About the same decrease occurred in the first determination after the resin had run dry, due to the resulting air pockets in the column.

Determination of Base Bound to Carbonic Acid.—We carried out some determinations with a solution 0.120N with respect to NaCl and 0.030N with respect to $NaHCO_3$. We found considerable losses of carbon dioxide, varying from 3 to 40 per cent, even when collecting the effluent in NaOH in flasks sealed by rubber stoppers with sodium hydroxide tube and when stirring with nitrogen during the titration of the excess NaOH. This must be due to adsorption of carbon dioxide by the resin.

We next ascertained whether heating of the effluents to just below the boiling point results in complete removal of all carbon dioxide. In determining the base content of a 0.120N NaCl solution and the solution 0.120N with respect to NaCl and 0.030N with respect to $NaHCO_3$ we found 119 (±0.7) meq. per liter and 119 (±1.0) meq. per liter, respectively.

Total Base Content of Urine.—The pH of urine may vary between 4.8 and 8.2, in contrast to the very small variations in the pH of serum. This necessitates the preliminary neutralization of the urine samples. The adopted procedure for urine was almost as accurate as the one for serum. The relative

standard deviations for serum and urine were 1.2 per cent and 1.7 per cent, respectively. The recovery for serum was 100.5 per cent and for urine 99.2 per cent.

It should be remarked that in this determination the ammonia concentration is included in the total base content of the urine.

Total Base Content of Tissues.—The difficult part in the total base determination in tissues proved to be ashing the sample and dissolving the ash quantitatively. The large series of determinations we had to carry out made it necessary to use porcelain crucibles instead of platinum ones. Ashing at high temperature (600 to 700° C.) made the ash adhere to the wall of the crucible with consequent incomplete dissolution in the dilute HCl. Ashing at low temperatures (below 500° C.) gave a carbonaceous ash with carbon particles occluding a marked amount of minerals. Silica crucibles gave no better results. Neither did addition of a drop of concentrated H_2SO_4 to the ash and subsequent heating on the Bunsen flame improve the dissolution of the ash. Wet ashing with HNO_3 and $HClO_4$ was also tried, but better results were not obtained. Most satisfactory results were obtained by ashing in an electric furnace at 500 to 550° C. for five to seven hours. The standard deviation of the total base determination in tissue with this ashing procedure was 6.9 per cent (for duplicate determinations in the same ash solution 1.3 per cent).

DISCUSSION AND SUMMARY

From the foregoing results it will be clear that reliable determinations with the ion exchange method are possible only if: (a) blank determinations are carried out to check if the columns have been washed sufficiently; (b) only samples having a total base concentration of 0.005 to 0.01N are percolated; (c) a low percolation rate (0.8 ml. per minute is used; (d) the resin is kept immersed by water before and during the percolation.

As at least 30 determinations can be carried out with the same column of resin before regeneration is necessary, it saves time and expense to use the same columns until they need regenerating instead of making new columns for every determination.

No reliable determination of base bound to carbonic acid is possible by cation exchange. Our method for serum determines only total base minus base bound to carbonic acid. Probably in the experiments of Gorter and Van Royen, who claim to determine the base bound to carbonic acid also, the losses of carbon dioxide were more or less compensated by the absorption of carbon dioxide by the alkali during collection of the effluent and subsequent titration.

It also proved to be possible to apply the method to urine and tissue ash. The determination in tissue ash requires the use of platinum crucibles for accurate work, due to the difficulties encountered in dissolving the ash quantitatively when using porcelain crucibles.

SUMMARY

A critical investigation of the total base determination in serum by ion exchange was carried out. Conditions necessary for obtaining reliable and

accurate results are given and a modified procedure is described. The method is extended to the determination of the total base content of urine and tissue ash.

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A METHOD OF SHELL FREEZING, UTILIZING THE STANDARD DENTAL MOTOR WITH A FLEXIBLE SHAFT

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MOST laboratories use a dental motor of some type for egg work and will have equipment on hand to shell freeze ampules by hand, either for storage in the deep freeze or for lyophilization.

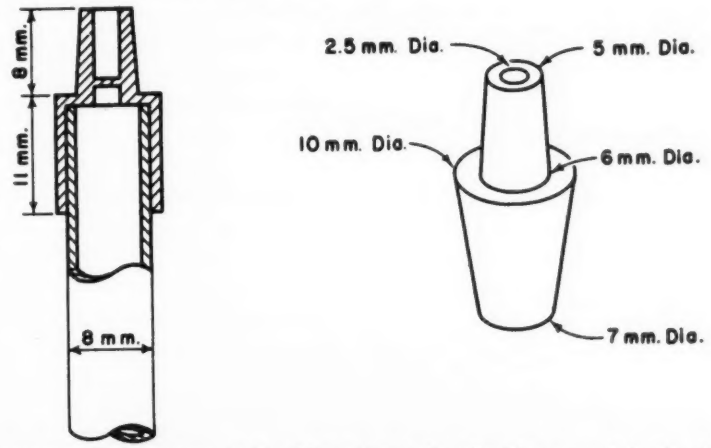

Fig. 1.—Diagram and measurements of rubber stopper for attaching ampule to chuck of flexible dental drill.

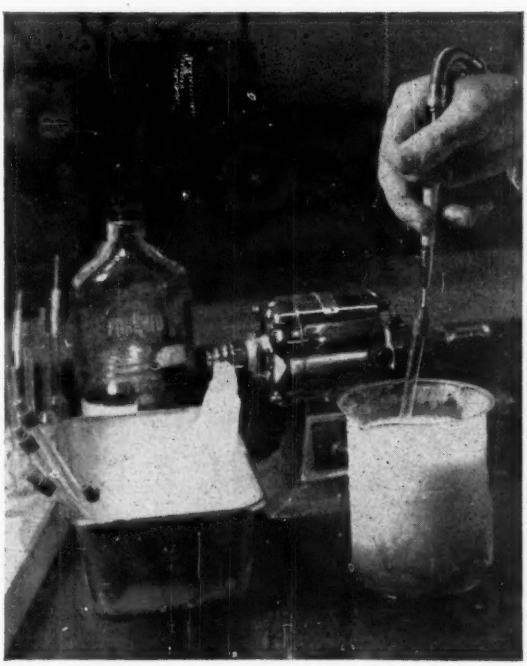

Fig. 2.—Photograph showing filled ampule being rotated in the carbon dioxide alcohol mixture by a flexible dental drill.

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This method consists of attaching a W-1 size sleeve-type rubber stopper with the sleeve over the neck end of the ampule after filling the ampule (Fig. 1). This may be done by using aseptic technique and sterile stoppers, with the neck of the ampule or the sleeve of the stopper wet with alcohol or sterile distilled water to aid in sliding the sleeve over the neck of the ampule.

With the ampule thus sealed, the material may now be shell frozen by inserting a 2.5 mm. bit, held in the chuck of the flexible drill, into the hole of the plug; rotating at a speed (200 r.p.m.) sufficient for the centrifugal force to throw the material against the sides of the ampules in a smooth and even film; then dipping vertically into a beaker of dry ice and alcohol mixture, as shown in Fig. 2.

Holding the drill and ampule vertically insures a smooth film which freezes within two to five seconds, depending on the thickness of the shell.

A few of the advantages of this method are that it is more rapid, and the material is evenly distributed and permits rapid lyophilization without melting if a vacuum of less than 1,000 microns is maintained. If the material is to be stored in the deep freeze refrigerator, the rubber stoppers are excellent seals.

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